Precision-cut liver slices as a model for intrinsic and idiosyncratic drug-induced liver injury

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Precision-Cut Liver Slices as a Model for Intrinsic and Idiosyncratic Drug-Induced Liver Injury

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General Introduction

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1. Introduction

An adverse drug reaction (ADR) is defined as "an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard for future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product" [1]. ADRs are a serious problem for public health and an obstruction to drug development and clinical use after marketing. For instance, almost half a million adverse drug events were reported worldwide in 1999 [2] and according to a study in the UK, ADRs are responsible for 1 in 15 hospital admissions, of which the mortality rate was 2.3% [3]. Additionally, from almost 550 drugs that were approved for the US market between 1975 and 1999, 10% of those acquired black box warnings and almost 3% were subsequently withdrawn from the marketas a result of ADR [4]. In addition to the suffering of the patients from this ADR, withdrawal of a drug means a massive financial loss to the pharmaceutical company that already spent up to a few billion dollars developing it.

Of all organs the liver is involved most frequently in ADRs as itis highly exposed to orally-ingested drugs and contains a high concentration of drug-metabolizing enzymes that may produce toxic metabolites. Drug-induced liver injury (DILI) has surpassed viral hepatitis as the leading cause (~50% of cases) of acute liver failure cases in the USand can lead to liver transplantation or death [5, 6]. The predominant clinical presentations of DILI resemble acute hepatitis or cholestatic liver disease, typically accompanied by markedly elevated serum transaminases, jaundice, systemic symptoms and in the more severe cases, coagulopathy and encephalopathy indicative of acute liver failure [7]. DILI has also been the top reason for withdrawing drugs from the market [4, 8].

The manifestation of DILI can be in the form of acute or cholestatic liver injury. Acute liver injury can involve two different modes of cell death; traumatic (necrosis) or programmed (apoptosis) cell death. The relative importance of these two modes of cell death is still arguable in DILI. When the injury is not massive, apoptosis might be less damaging than necrosis, which is more likely to promote inflammation and consequent collateral damage [9]. The ATP level can determine whether injury leads to apoptosis or necrosis. Conditions in which a mild damage to mitochondria occurs but ATP level is still sustained above a critical threshold, will favor apoptosis. Meanwhile, a more severe hit to mitochondria might favor necrosis [10]. Moreover, cholestatic liver injury refers to liver injury in the presence of bile flow impairment and is characterized by increased hepatocellular levels of toxic bile acids [11].

DILI, which has not been detected in preclinical studies, can be observed in clinical trials when it occurs at an incidence of up to one in several hundreds or thousands patients.

However, when the incidence is below one in ten thousands of patients DILI can be observed only after a drug is on the market, as this incidence is too low to be detected during clinical trials [12]. Therefore, DILI is commonly classified into intrinsic (type A) vs. idiosyncratic (type B) hepatotoxicity. Intrinsic DILI is regarded predictable (relatively higher incidence and dose-dependent), while idiosyncratic DILI is usually unpredictable (very low incidence and no obvious dose-dependency). One common misconception is that a drug can be clearly classified as either intrinsic or idiosyncratic hepatotoxin, as some drugs are known to cause both.One example is isoniazid that is associated with mild intrinsic DILI as well as severe idiosyncratic DILI[13]. The difference between these two types will be described in detail in the following sections.

2. Intrinsic DILI

Intrinsic DILI is usually unrelated to the pharmacology of the drug, exhibits dose and time dependency and can often be detected during pre-clinical (at least as species differences are minimal) or clinical trials. Paracetamol or acetaminophen (APAP) is considered as a classic intrinsic hepatotoxin that may cause severe liver toxicity above therapeutical doses. Approximately 80% of drug-associated cases of liver failure is caused by APAP, be it intentional or accidental overdose [5], but other well-known examples are methotrexate[14], isoniazid[15], and voriconazole[16, 17]. APAP overdose also causes severe hepatotoxicity in experimental animals,but the dose that exerts the toxicity highly depends on the species. Experiments in primary mouse, rat, and human hepatocytes indicated that mouse is the most sensitive species, while human is the least sensitive species to APAP toxicity[18]. Since APAP is the most widely-studied drug in the field of toxicology, there is a myriad of data about the mechanisms of APAP-induced liver injury that might be beneficial in explaining the mechanisms of intrinsic DILI in general.

APAP is commonly used as a painkiller (analgesic) and/or to reduce fever (antipyretic). APAP is generally safe to use at a dose below 4 g per day. It is believed that APAP by itself is not cytotoxic to the liver[19]; however APAP is metabolized extensively by the liver via three main pathways, namely glucuronidation, sulfation and oxidation[20]. The first two pathways lead to formation of non-toxic conjugates (APAP-glucuronide and APAP-sulfate) and are quantitatively the most important, but the oxidative, CYP-dependent pathway (mainly involving CYP 2E1 and 3A4) is the primary culprit as far as hepatotoxicity is concerned[21]. This pathway produces a toxic reactive metabolite known as *N*-acetyl-*p*-benzo-quinoneimine (NAPQI), which at a low level, can normally be detoxified by conjugation with glutathione (GSH) which is present in high concentration in the liver, forming APAP-GSH[22]. APAP-GSH is the initial sulfur-containing metabolite of APAP produced by

the liver but this complex is converted to its non-toxic metabolic breakdown products, namely cysteine (APAP-CYS) and N-acetylcysteine (APAP-NAC) conjugates, which are subsequently eliminated via the urine. Therefore, little, if any, APAP-GSH is found in the urine of intact animals[23]. In human adults, the glucuronide conjugate accounts for 60-65%, the sulfate for 30-35% and the oxidative conjugates (APAP-GSH, APAP-CYS, APAP-NAC) for ca. 5% of the metabolites found in the urine[24].

At the normal therapeutic dose, the level of NAPQI is low enough to be detoxified by conjugation with GSH. However, after an overdose resulting in a massive production of NAPQI due to saturation of the other metabolic pathways, hepatic GSH is depleted and can no longer prevent toxicity, leading to the binding of NAPQI to proteins. Covalent binding to critical proteins has been postulated to be the one of the mechanisms of APAP toxicity[25]. Reactive metabolites of APAP were found to bind extensively to mitochondrial proteins in mouse liver[26].

In addition, APAP and/or NAPQI are known to activate a transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) in mouse *in vivo*[27] and *in vitro*[28], indicating that these compounds may induce oxidative stress. The mammalian oxidative stress response is coordinated by Nrf2, which activates the transcription of antioxidant genes[29, 30]. Under normal conditions, Nrf2 is held inactive in the cytoplasm by a protein known as Kelch-like ECH-associated protein-1 (Keap1), which negatively regulates Nrf2 activity[29]. *Nrf2*-deficient mice exhibit an increased sensitivity to APAP, resulting in greater severity in hepatic damage and increased lethality[31, 32], while activation of Nrf2 by oleanolic acid protects against APAP-induced hepatotoxicity[33]. Therefore, Nrf2 is generally considered to serve as an endogenous regulator by which cells combat oxidative stress, which also plays a role after APAP-induced hepatotoxicity.

Additionally, disturbance of mitochondrial function is also considered as an important mechanism of DILI [13, 34]. APAP administration was known to deplete mitochondrial glutathione and to alter mitochondrial membrane permeability thereby decreasing the efficiency of oxidative phosphorylation [35]. APAP andmore likely NAPQI are hypothesized to cause mitochondrial permeability transition (MPT), i.e. opening of the 'MPT pore' located in their inner membrane. This renders mitochondria more susceptible to oxidative stress damage, especially during increased reactive oxygen species (ROS) production, which leads to apoptosis, and if coupled with the loss of adenosine triphosphate (ATP), eventually to necrosis [13, 36]. APAP-induced glutathione depletion and mitochondrial dysfunction have been associated with c-Jun N-terminal kinase (JNK) pathway activation and translocation of JNK to mitochondria where it induces mitochondrial permeability transition [37].

3. Idiosyncratic DILI

Idiosyncratic DILI is defined as DILI occurring only in a small fraction (< 1:10,000)of the patients taking therapeutical doses of a drug, but it is responsible for ~13% of acute liver failure cases in the US [38]. It is typically not related to the pharmacological effects of the drug and does not have an obvious dependence to dose or time. Intrinsic DILI normally occurs during drug overdose, while idiosyncratic DILI can occur when patients taking normal therapeutical doses of a drug. However, drugs that are administered at doses lower than 10 mg/day are generally not associated with idiosyncratic DILI, while the typical idiosyncratic DILI-causing drugs are given at higher doses (>100 mg/day) [39]. Another important characteristic of idiosyncratic DILI is a delay (typically 5-90 days) between the drug administration and the onset of the adverse reactions [40, 41]. In general, there are two widely-accepted categories of idiosyncratic hepatotoxicity, i.e. immunologic idiosyncrasy and metabolic idiosyncrasy. Immunologic idiosyncrasy is associated with the presence of signs of drug hypersensitivity, such as fever, rash, eosinophilia, antidrug antibodies, etc. In contrast, metabolic idiosyncrasy refers to injury in the absence of these signs[42, 43].Drugs causing idiosyncratic DILI are very different compounds and they represent many pharmacological classes.For an overview on drugs that cause idiosyncratic liver injury the reader is referred to a review by Kaplowitz [44].

Apart from these observed characteristics, relatively little definitive evidence exists that can explain the mechanisms of idiosyncratic DILI. There are several factors, including drug properties, genetic variation, and environmental factors, which are likely to play a role in idiosyncratic DILI simultaneously [8, 45]. Many idiosyncratic DILI cases involve the biotransformation of drugs into reactive metabolites [46, 47], but not all idiosyncratic DILI-associated drugs produce reactive metabolites [48].Several polymorphisms in drug metabolizing enzymes are associated with higher prevalence of idiosyncratic DILI[49]. It is also believed that some idiosyncratic DILI has an association with specific human leukocyte antigen (HLA) genotypes. In amoxicillin-clavulanic acid (AC)-induced hepatitis, 57% of affected patients possess a specific HLA haplotype, while the prevalence of this haplotype in the general population is only 12%[50]. However, even though it would be useful to study the pathogenesis of AC-DILI in relation to these HLA genotypes, their role as biomarkers is limited due to the low positive predictive value [51]. Moreover, polymorphisms in genes encoding cytokines were shown to influence a person's susceptibility to hepatotoxicity. Genes that are associated with high IL-4 and low IL-10 expression were found to be risk factors for diclofenac-induced liver toxicity [52]. There are many other risk factors associated with idiosyncratic DILI, such as age, sex, and weight. For idiosyncratic DILI in adults, older age is generally associated with cholestatic damage with male prominence [51] and

hepatocellular DILI injury is more common among women [38]. A specific example is the higher prevalence of halothane-induced hepatitis in women and obese patients [53].

Several mechanistic hypotheses have been proposed that may explain idiosyncratic DILI:

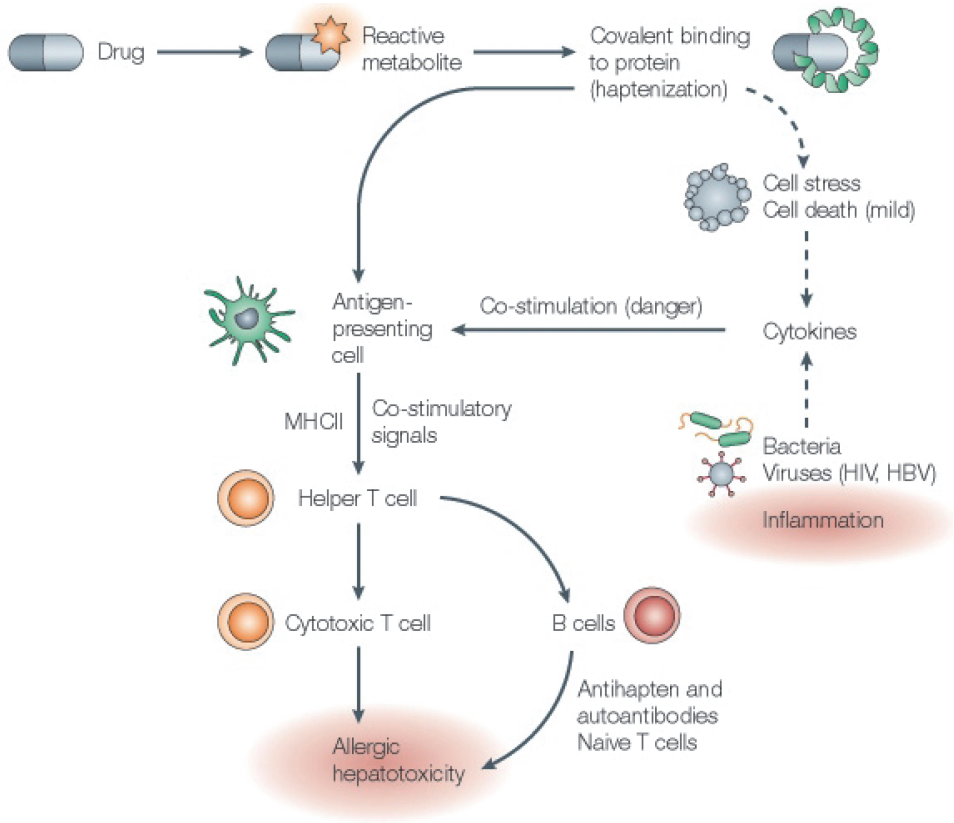

Figure 1. Illustration of the Danger Hypothesis.This figure is republished with permission[44].

3.1. Danger Hypothesis

Many idiosyncratic DILI reactions are mediated by adaptive immune responses[54, 55]. A small molecule, such as a chemically-reactive metabolite, cannot induce immune response unless it is bound to a protein; such a small molecule is then called a hapten [56]. This hapten-protein complexformation modifies the protein, which is then seen as a foreign antigen that initiates an immunological response against this protein(primary signal) [57]. However, without the presence of an adjuvant that stimulates the antigen presenting cells (APCs), this foreign antigen does not induce a substantial immune response and therefore is insufficient to cause injury [58, 59]. A secondary 'danger' signal, which might be mild

cell death or aconcomitant inflammation, is needed to precipitate the primary reaction, activating the helper T cells leading to a T-cell response to the antigen. This activation then triggers the effector lymphocytes, cytotoxic T cells and B cells, leading to immune-mediated idiosyncratic DILI [44]. The danger hypothesis is illustrated in Figure 1.

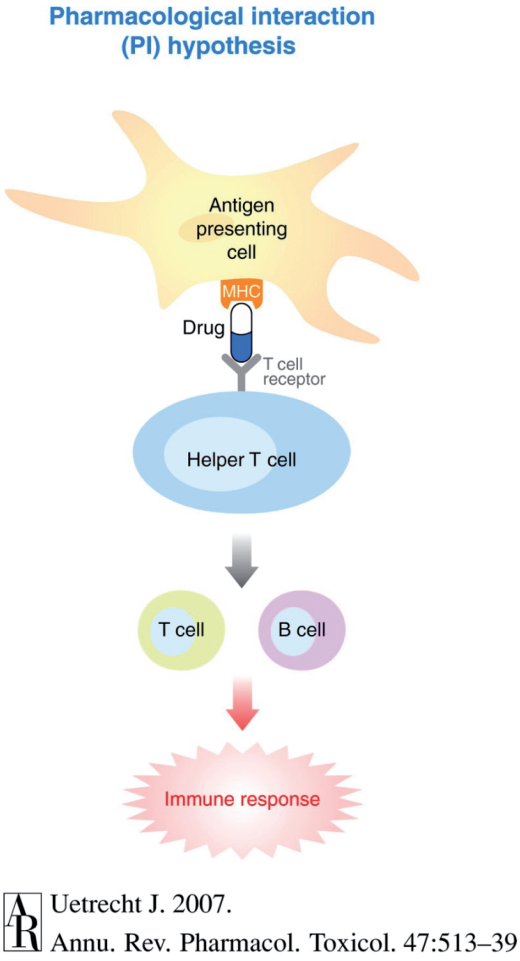

Figure 2. Illustration of the PI Hypothesis.This figure is republished with permission[54].

3.2. Pharmacological Interaction (PI) Hypothesis

Some parent drugs themselves, without metabolism into reactive metabolites and further processing process, can initiate immune responses by binding reversibly to the complex of major histocompatibility complex (MHC) on APCs and T cell receptor on helper T cells. This is referred to as the PI hypothesis because the drug acts more as a pharmacological

agent [60]. One example is sulfamethoxazole, which is not chemically reactive per se, but it can bind to human T-cell clones via direct, MHC-dependent manner [61].Unlike the danger hypothesis, a secondary signal is not includedin this hypothesis. However, the PI and danger hypotheses might not be mutually exclusive as a reactive metabolite can act as the danger signal, by binding to the MHC - T cell receptor complex without serving as a hapten that is presented by the MHC[55].The PI hypothesis is illustrated in Figure 2.

3.3. Multiple Determinant Hypothesis

According to the multiple determinant hypothesis, the low occurrence of idiosyncratic DILI might be explained by the requirements of four critical events to occur simultaneously. The determinants of these critical events are: chemical properties (formation of reactive metabolites, interaction with other drugs); exposure (drug concentration in the plasma and/or liver), environmental factors (pre-existing diseases, stress levels, food and alcohol consumption), and genetic factors (gene polymorphisms). Each event has an independent probability of occurring, but all of them are required to precipitate an idiosyncratic DILI. Therefore, the probability for the occurrence of idiosyncratic DILI is the product of the probabilities of each independent event, which may explain its low incidence. Based on this hypothesis, an individual would suffer idiosyncratic DILI when exposed to a drug with a potential to cause idiosyncratic DILI and when the exposure level, environmental, and genetic factors all simultaneously favor the expression of the toxicity [62].

3.4. Inflammatory Stress Hypothesis

Inflammation is traditionally defined as a complex biological response of tissues to harmful stimuli, such as injury, infection, or irritation, characterized by the presence of redness, swelling, pain, heat and function loss. In addition, it can also be viewed from the perspectives of innate immune system cell activation, actions of the mediators produced, and changes in inflammatory gene expression and cell signaling [63]. Inflammation involves not only traditional inflammatory cells (e.g. macrophages, neutrophils) and the mediators they produce (e.g. cytokines / chemokines), but also the endothelial cells and parenchymal cells in the tissue. Inflammatory episodes, such as those caused by bacterial or viral infection and intestinal microbial disturbances, are common in humans and animals [64].

It was suggested that an acute episode of inflammation can interact with concurrent drug therapy to precipitate idiosyncratic DILI. This modest inflammatory stress can lower the threshold for drug toxicity, thereby shrinking the therapeutic window resulting in a toxic response at an otherwise safe dose of the drug. Alternatively, a drug might also augment a mild inflammatory reaction rendering it injurious[65, 66]. These inflammatory

episodes can be caused by inflammagens, such as polyinosinic:polycytidylic acid (poly I:C) and lipopolysaccharide (LPS). Poly I:C is a synthetic double-stranded RNA that is used experimentally to model viral infections *in vivo*, while LPS is a component of the cell walls of gram-negative bacteria that is capable of inducing inflammation *in vivo.* Poly I:C and LPS activate numerous inflammatory pathways via activation of Toll-like receptors 3 and 4 respectively [67, 68]. These include activation of cellular signaling pathways, transcription factors (e.g. p38, nuclear factor κB), and inflammatory mediators (e.g. cytokines, chemokines), which in turn activate innate immune cells (e.g. monocytes, neutrophils), coagulation, and complement systems, eventually leading to liver injury [63]. Even though different inflammatory mediators might be involved in injury by different drugs, neutrophils and tumor necrosis factor (TNF) were found to be critical mediators of injury in many idiosyncratic DILI models based on this hypothesis [69-71]. This hypothesis does not exclude the involvement of reactive metabolites and in fact can be valid for several mechanisms of DILI, including those that involve reactive metabolites.

4. Experimental Models of idiosyncratic DILI

It was estimated that to detect idiosyncratic DILI that occurs in 1 in 10,000 patients, approximately 30,000 patients are required in clinical testing [6]. Assuming a similar incidence in animals, 30,000 animals are needed for toxicity testing to detect idiosyncratic DILI. Since such large studies are not feasible, better experimental models to predict idiosyncratic DILI need to be developed. According to Shenton and Roth, the most useful idiosyncratic DILI experimental models have liver injury as the major endpoint with identical mode of injury as in human, can distinguish idiosyncratic DILI-related drugs from those that are not, and are reproducible in a large fraction of the subjects in the study [72, 73].

4.1. *In vivo* models

Both in the danger hypothesis and the inflammatory stress hypothesis, an inflammation is involved in the precipitation of the idiosyncratic toxicity of a drug. The research group of Robert Roth has been the pioneer to develop rodent models that can predict idiosyncratic DILI based on the inflammatory stress hypothesis [69-71, 74-76].Several human idiosyncratic DILI-causing drugs have been shown to also cause liver injury in rats and mice *in vivo* when coupled with a nontoxic dose of an inflammagen. The use of a low dose of inflammagen, such as LPS, is particularly important in developing an idiosyncratic DILI model in animals as exposure to large amount of LPS by itself can also result in liver injury, as well as death [77]. Several examples ofcotreatment of rodents with LPS and idiosyncratic DILI-causing drugs resulting in hepatotoxicity will be described further. These studies revealed that the

drug dose and the interval between LPS and drug administration are crucial to obtain a hepatotoxic response and the optimal dose and interval is different for each drug [73].

Cotreatment of rats with LPS and chlorpromazine increased the activities of liver transferases and serum creatine kinase, which is associated with human idiosyncratic reactions, when compared to the treatment of LPS or chlorpromazine alone [74]. The hepatotoxicity of monocrotaline (MCT) in rats was also augmented by LPS and the mortality rate was much higher than controls when LPS was given one hour before MCT administration [69]. Neutrophil depletion protected against liver injury caused by the cotreatment of rats with LPS and diclofenac (DF) [75] or trovafloxacin (TVX) [70]. Ranitidine (RAN) treatment caused the LPS-induced increase in serum TNF concentration to last longer than in rats given LPS alone and LPS/RAN-cotreated rats developed hepatotoxicity [78]. Moreover, TNF and plasminogen activator inhibitor-1 independently contributed to neutrophil activation, which is critical to the hepatotoxicity caused by LPS and sulindac cotreatment in rats [76, 79].

In addition to the rat models described above, mouse models were also developed for Idiosyncratic DILI. LPS renders mice more sensitive to the hepatotoxicity caused by TVX and this liver injury appeared to be dependent on TNF and interferon gamma (IFN-γ). However, coexposure of LPS and levofloxacin, another fluoroquinolone antibiotic without idiosyncratic DILI-properties, did not result in hepatotoxicity [71, 80]. Coexposure of halothane-treated male mice to lipopolysaccharide exacerbated their hepatotoxic response similar to the response observed in halothane-treated female mice [81]. This finding is consistent with the fact that women are more sensitive to halothane-induced hepatitis [53].

From these animal models, we learned that it was possible to predict the propensity of drugs to cause idiosyncratic DILI in humans and these drugs can also be distinguished from their comparator drugs that do not cause idiosyncratic DILI. The mechanisms of the synergistic toxicity observed in these models appeared to be dependent on neutrophils and TNF for many idiosyncratic DILI drugs.

4.2. *In vitro* models

The most important questions that one should ask when developing animal models for toxicity is how predictive are these models for human toxicity and how similar are the mechanisms of toxicity of a compound elucidated by these animal models when compared to the real situations in humans. It is well known that generally the concordance of xenobiotic toxicity in human and animals is alarmingly low. Out of 150 compounds tested with human toxicity, concordance was seen in 63% of non-rodent studies (primarily dog) and 43% of rodent species (primarily rat). Mouse was actually the least predictive species for human toxicity out of 5 species tested. Despite its relatively high incidence in all

species, hepatotoxicity in human was surprisingly poorly predicted from animal studies [82]. One major advantage of using *in vitro* models is the possibility of working with cells from human instead of animal origin. Additionally, *in vitro* models employing animal cells are also useful to make direct comparison to the human *in vitro* data. Another advantage is that the use of animal and human in vitro models contributes to the refinement, reduction, and eventually replacement of animal studies.

Several *in vitro* models predicting idiosyncratic DILI based on the inflammatory hypothesis have been developed. In 2006, Tukov *et al.* utilized a rat Kupffer cell-hepatocyte coculture system to model inflammagen-drug interactions *in vitro*. It was found that MCT alone had no effect on TNF release, but it elevated LPS-induced TNF release in these cocultures[83]. These results were in line with the observations from *in vivo* experiments, in which MCT did not increase plasma TNF concentration when given to rats by itself, but augmented and prolonged the increase in plasma TNF concentration in LPS-cotreated rats [69]. Thus, the *in vitro* results from Kupffer cell-hepatocyte coculture qualitatively mirror reported data from whole animal studies.

Since LPS cannot induce an inflammatory reaction in pure hepatocyte cultures, pro-inflammatory cytokines, such as TNF, were used instead to induce inflammation-like reactions. TNF was found to enhance the cytotoxicity of the metabolite sulindac-sulfide in rat primary hepatocytes and also in HepG2 cells, a human liver carcinoma cell line [84]. Similarly, TNF significantly enhanced DF-induced apoptosis in HepG2 cells, whereasTNF had no effects on another structurally different non-steroidal anti-inflammatory drug, naproxen. It was found that the DF/TNF-induced apoptosis was dependent on caspase-8, caspase-3, and JNK activities and that DF inhibited the TNF-induced nuclear factor-κB activation [85].

Cosgrove et al. performed experiments cotreating HepG2 cells as well as primary human and rat hepatocytes with LPS and a cytokine mix (TNF, IFN-γ, interleukin (IL) 1α, and IL 6) and idiosyncratic DILI drugs (TVX, RAN, nefazodone, nimesulide) and found synergistic induction of hepatocellular death. However, this synergistic hepatotoxicity was not observed with the use of the corresponding comparator drugs that are not associated with idiosyncratic DILI (LVX, cimetidine, buspirone, aspirin) instead [86]. The intrinsic drawback of the use of HepG2 cells for prediction of Idiosyncratic DILI is the well-known fact that these cells represent hepatoma cells and have lost most of their phase I and part of their phase II metabolism capacity[87, 88]. Therefore, although it is a useful model to test intracellular mechanisms of cell injury including the elucidation of cell signaling pathways, it may be difficult to detect metabolism-related toxicity.

An extensive in vitro approach was recently developed by Thompson *et al.*to assess the idiosyncratic DILI risks for 36 known drug candidates. The approach involved several in vitro

assays, which quantified the toxicity of the drugs in three different cell lines (representing toxicities caused bythe parent drug, the possible reactive metabolite, and mitochondrial injury) as well as the inhibitions of the human bile salt export pump (BSEP) and the rat multidrug resistance associated protein 2 (Mrp2). Additionally, the covalent binding burden of each drug was also estimated. Combination of these aggregated results led to the discrimination of 27 drugs that had marked idiosyncratic DILI concern and 9 that had low concern of idiosyncratic DILI with high rates of specificity (78%) and sensitivity (100%) [89].

4.3. Potential *ex vivo* models

Precision-cut liver slices (PCLS) have been receiving increased attention as a robust *ex vivo* tool to study drug metabolism and toxicity. One definite advantage of the PCLS system over other *in vitro* models is that a PCLS contains all liver cell types in their natural ratio and extracellular matrix environment, retaining normal tissue architecture and cell-to-cell and cell-to-matrix interactions. PCLS demonstrate higher similarity to intact liver compared to primary hepatocytes and cell lines based on the gene expression profiles [90]. They possess a complete set of active phase I and II drug metabolism enzymes and many experiments related to drug metabolism have been performed in PCLS [91-96]. Moreover, microarray analysis of xenobiotic-treated rat PCLS showed that they could mimic the toxicity as observed *in vivo* and discriminate between different mechanisms of hepatotoxicity [97]. A relatively stable expression of genes involved in drug transport, metabolism, and toxicity was reported for normal incubationof human PCLS for 24h, whereas incubation with APAP caused significant changes in these gene expression[98]. It has also been shown that the Kupffer cells in PCLS can be activated by LPS treatment inducing an inflammatory response as evidenced by the production of nitric oxide (NO) and inflammatory cytokines, such as TNF, IL-1β, and IL-6 [99-102]. Despite extensive use of this PCLS system in drug metabolism and intrinsic toxicity studies, it has never been used to study idiosyncratic hepatotoxicity before.

5. Aim and Outline of the Thesis

The research of this thesis was focused on the use of PCLSfor the development of an *ex vivo* model for intrinsic and idiosyncratic DILI. One main objective was to compare and contrast the findings in PCLS from animals and humans. Therefore, PCLS from three different species, namely, mouse, rat, and human were utilized in the experiments. The goal of this research is to contribute to a better early prediction of drugs that cause intrinsic or idiosyncratic toxicity in humans before the drug is released on the market with concurrent reduction in the use of experimental animals.

In **chapter 2**, the toxicity of the intrinsic hepatotoxin, APAP and its regioisomer, N-acetyl-meta-aminophenol (AMAP) was compared in mouse, rat, and human PCLS by means of ATP content and histomorphology. AMAP possesses similar analgesic and antipyretic properties as APAP and has always been described as a non-toxic isomer of APAP. AMAP was shown to be non-hepatotoxic in mouse *in vivo* and *in vitro* as well as in hamster *in vivo*[26, 103-105]. The profiles of the proteins released into the media of mouse and rat PCLS after exposure of APAP and AMAP were alsoanalyzed. Additionally, the metabolites of APAP and AMAP in PCLS media from the three species were determined in order to investigate whether species difference in the metabolite production could explain the species difference in toxicity.

Chapter 3 focused on the profiling of the proteins in the media of PCLS after treatments with several hepatotoxins. The protein profiles following APAP, AMAP, DF, or LPS treatments in mouse, rat, and human PCLS were compared. One potential marker associated with DILI is the iron-regulating hormone hepcidin, which was decreased in the plasma and livers of mice with APAP-induced liver injury as a result of oxidative stress[106]. Therefore,we also measured the concentration of hepcidin in the medium of mouse, rat, and human PCLS after treatments with these hepatotoxins. Extrapolation of the *ex vivo* data to *in vivo* data was performed by comparing the protein profiles of mouse PCLS medium and mouse urine and liver homogenates after APAP treatment.

The aims of the studies described in the next two research chapters were to test if PCLS can be a suitable *ex vivo* model to study the inflammatory stress hypothesis, whether idiosyncratic DILI-associated drugs could be identified with this model and to discover biomarker(s) that can identify drugs as potentially inducing idiosyncratic DILI.In both chapter 4 (mouse) and chapter 5 (human), the toxicity of five drugs known to cause idiosyncratic DILI in humans: DF, ketoconazole (KC), clozapine (CZ), carbamazepine (CBZ), and troglitazone (TGZ) were investigated in the absence and presence of LPS in mouse and human PCLS. Additionally, comparator drugs that are in the same chemical class of drugs as KC and CZ and are known not to cause idiosyncratic DILI, i.e. voriconazole (VC) and olanzapine (OZ) respectively, were also investigated. The viability of PCLS was determined by means of ATP content and histomorphology. In mouse PCLS, leakage of lactate dehydrogenase was measured as an extra viability test. Aiming to find biomarkers that are associated with idiosyncratic liver injury, we also measured the levels of reduced and total glutathione in the PCLS and the release of several cytokines in the medium of PCLS. Furthermore, each drug's effect on the LPS-induced release of these cytokines was examined. The effects of each drug on LPS-induced nitric oxide release in mouse PCLS and on LPS-induced soluble intercellular adhesion molecule-1 release in human PCLS were also

investigated. Additionally, it was also of our interest to compare and contrast the effects of interaction between LPS and these drugs in mouse and human PCLS to investigate the species differences in toxicity.

Finally, a summary, discussion and future directions of the use of PCLS to study intrinsic and idiosyncratic DILI are presented in chapter 6.

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2

AMAP, the alleged non-toxic isomer of acetaminophen, is toxic in rat and human liver

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Archives of Toxicology: in-press

Abstract

N-acetyl-meta-aminophenol (AMAP) is generally considered as a non-toxic regioisomer of the well-known hepatotoxicant acetaminophen (APAP). However, so far AMAP has only been shown to be non-toxic in mice and hamsters. To investigate whether AMAP could also be used as non-toxic analog of APAP in rat and human, the toxicity of APAP and AMAP was tested *ex vivo* in precision-cut liver slices (PCLS) of mouse, rat and human. Based on ATP content and histomorphology, APAP was more toxic in mouse than in rat and human PCLS. Surprisingly, although AMAP showed a much lower toxicity than APAP in mouse PCLS, AMAP was equally toxic as or even more toxic than APAP at all concentrations tested in both rat and human PCLS. The profile of proteins released into the medium of AMAP-treated rat PCLS was similar to that of APAP, whereas in the medium of mouse PCLS it was similar to the control. Metabolite profiling indicated that mouse PCLS produced the highest amount of glutathione conjugate of APAP, while no glutathione conjugate of AMAP was detected in all three species. Mouse also produced ten times more hydroquinone metabolites of AMAP, the assumed proximate reactive metabolites, than rat or human. In conclusion, AMAP is toxic in rat and human liver and cannot be used as non-toxic isomer of APAP. The marked species differences in APAP and AMAP toxicity and metabolism underline the importance of using human tissues for better prediction of toxicity in man.

1. Introduction

In studies on the mechanism of toxicity of a xenobiotic, a non-toxic analog with a pharmacological activity similar to that of the toxic compound is frequently taken as control to distinguish pharmacological from toxicological effects.Based on studies in mice and hamsters, N-acetyl-meta-aminophenol (AMAP), is generally considered as a non-toxic comparator drug for acetaminophen (N-acetyl-para-aminophenol, APAP). However, it is also known that the concordance of xenobiotic toxicity in human and animals is alarmingly low. Out of 150 compounds tested with human toxicity, concordance was seen in 63% of non-rodent studies (primarily dog) and 43% of rodent species (primarily rat). Mouse was actually the least predictive species for human toxicity out of 5 species tested. Despite its relatively high incidence in all species, hepatotoxicity in human was surprisingly poorly predicted from animal studies [1]. But no data is available on the toxicity of AMAP in the human liver.

APAP is a frequently used analgesic and antipyretic agent and is considered to be safe at therapeutic doses (≤4g per day). However, APAP overdose may cause severe hepatotoxicity that leads to liver failure and death in both man and laboratory animals. N-acetyl-p-benzo-quinone imine (NAPQI), an electrophilic and oxidative reactive metabolite formed by cytochrome P450s, is commonly thought to be primarily responsible for the toxic effects of APAP [2]. At therapeutic doses, this metabolite is efficiently detoxified by glutathione (GSH) conjugation. However, after an overdose, hepatic GSH is depleted and can no longer prevent accumulation of NAPQI, leading to its binding to cysteine-sulfhydryls on proteins. The mechanism of APAP-induced hepatotoxicity in humans and mice involves covalent binding to critical proteins [3], mitochondrial dysfunction and nuclear DNA fragmentation [4]. Furthermore, APAP and/or NAPQI are known to induce oxidative stress and thereby activate the nuclear factor erythroid 2-related factor 2 (Nrf2), which may serve as an endogenous regulator by which cells combat oxidative stress *in vivo*[5] and *in vitro*[6].

AMAP, the regioisomer of acetaminophen, possesses similar analgesic and antipyretic properties as APAP and was shown to be non-hepatotoxic at doses where APAP showed overt toxicity in mouse *in vivo* and *in vitro*[7, 8, 8, 9] as well as in hamster *in vivo*[10]. In mouse hepatocyte cultures, AMAP was cytotoxic only at 10-fold higher concentration than APAP [11]. In contrast to APAP, little GSH depletion was observed after the administration of AMAP to mice [7]. However, pre-treatment of mice with buthioninesulfoximine, which inhibits the synthesis of GSH, rendered the mice more susceptible to hepatotoxicity after AMAP administration [12]. Additionally, the levels of covalent binding of AMAP were comparable to that of APAP in mouse [13] or hamster [10]. AMAP was also shown to have effects on the mouse liver proteome, which was qualitatively similar to that of APAP [14]. Despite all these

characteristics, AMAP is still widely considered as a non-hepatotoxic analog of APAP [15], even though so far AMAP has only been studied in two species, mouse and hamster.

As we were interested to use AMAP as a non-toxic analog in our studies on the mechanism of APAP toxicity in rat and human liver, we investigated the hepatotoxicity of both APAP and AMAP in mouse, rat and human precision-cut liver slices (PCLS). PCLS retain the normal tissue architecture of an intact liver with all its cell types in their natural arrangement and intact intercellular interactions [16-18]. The gene expression profile of PCLS was shown to have a higher similarity to intact liver compared to primary hepatocytes and cell lines [19]. Moreover, microarray analysis of toxin-treated rat PCLS showed that they could correctly predict the hepatotoxicity as observed *in vivo* and discriminate between different mechanisms of toxicity [20]. Relatively stable expression of genes involved in drug transport, metabolism and toxicity during normal incubation of human PCLS for 24h was reported, whereas incubation with APAP caused significant changes in gene expression [21]. All phase I and II xenobiotic metabolism pathways are active in PCLS and their metabolism and toxicity functions correlate well with *in vivo* data [18, 22-26].

In the present study, we compared the toxicity of APAP and AMAP in mouse, rat and human PCLS by means of ATP content and histomorphology. Proteins released into the media of mouse and rat PCLS were also profiled. Additionally, the metabolites of APAP and AMAP in PCLS media from the three species were determined in order to investigate whether species difference in the metabolite production could explain the species difference in toxicity.

2. Materials and methods

2.1. Chemicals

APAP, AMAP, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Stock solutions of APAP and AMAP were made in DMSO (VWR, Briare, France) as the solvent.

2.2. Animals

Female C57BL/6 mice weighing 20-24 g and male Wistar rats (HsdCpb:WU) weighing 300-350 g were obtained from Harlan (Horst, the Netherlands). The mice and rats were housed on a 12-h light/dark cycle in a temperature- and humidity-controlled room with food (Harlan chow no 2018, Horst, the Netherlands) and tap water ad libitum. The animals were allowed to acclimatize for at least seven days before experimentation. The experimental protocols were approved by the Animal Ethical Committee of the University of Groningen.

2.3. Human liver tissue

Pieces of human liver tissue were obtained from patients undergoing partial hepatectomy (PH) for the removal of carcinoma or from liver tissue remaining as surgical waste after split liver transplantation (TX), as described previously [21]. The characteristics of these human livers are described in Table 1. The experimental protocols were approved by the Medical Ethical Committee of the University Medical Center Groningen.

2.4. Excision of mouse and rat liver

Under isofluorane/O_2 anesthesia, the liver was excised and placed into ice-cold University of Wisconsin (UW) organ preservation solution (DuPont Critical Care, Waukegab, IL, USA).

2.5. Preparation of mouse, rat and human PCLS

PCLS were made as described previously [27]. In brief, cylindrical cores were prepared by drilling a hollow metal tube (diameter = 5mm) into the liver. These cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs-Henseleit buffer saturated with carbogen (95% O_2 and 5% CO_2). PCLS (5 mm diameter, 200-300 μm thick and 5 mg wet weight) were stored in ice-cold UW solution until incubation.

2.6. Incubation of mouse, rat and human PCLS

Incubation of PCLS in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria) was performed as described before [27]. In brief, PCLS were pre-incubated at 37°C for 1 h individually in 1.3 ml Williams' medium E with glutamax-1 (Gibco, Paisley, UK), supplemented with 25 mM D-glucose and 50 μg/ml gentamicin (Gibco, Paisley, UK) (WEGG medium) in a 12-well plate with shaking (90 times/min) under saturated carbogen atmosphere. Pre-incubation allows the PCLS to restore their ATP levels. After pre-incubation, PCLS were transferred to fresh WEGG medium with different concentrations of APAP or AMAP or the vehicle, DMSO (final concentration of DMSO during incubation ≤0.5%) and incubated for 24h.

2.7. ATP and protein content of PCLS

Viability of mouse (n=3), rat (n=4) and human (n=5) PCLS after 24h incubation with APAP, AMAP or solvent control was determined by measuring the ATP content of the PCLS according to the method described earlier [27]. In brief, at the end of 24h incubation, three replicate PCLS were collected individually in 1 ml 70% ethanol

(v/v) containing 2 mM EDTA (pH 10.9) and snap-frozen in liquid nitrogen and stored at -80°C until analysis. The samples were homogenized using a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA) and centrifuged for 3 minutes at 13000 rpm and 4°C. The supernatant was diluted 10 times with 0.1 M TrisHCl containing 2 mM EDTA (pH 7.8) to reduce the ethanol concentration. The ATP content of the supernatant was measured using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) in a black 96-well plate Lucy1 luminometer (Anthos, Durham, NC, USA) using a standard ATP calibration curve.

Table 1. Human liver donor characteristics used in this study (n=5-6 for each endpoint; A = ATP, H = histomorphology, M = metabolism), PH is liver tissue after partial hepatectomy, TX is liver tissue remaining from donor liver after transplantation. The ATP content of human PCLS after 24h of incubation is indicated.

Liver id.	PH/TX	Sex	Age	ATP at 24h (nmol/mg protein)
A1	PH	male	80	9.7
A2	PH	male	59	7.1
A3	PH	male	64	4.7
A4	PH	male	65	6.2
A5	TX	male	10	11.1
H1	PH	female	76	9.9
H2	PH	female	62	4.0
H3	TX	male	27	8.4
H4	PH	male	57	6.0
H5	PH	male	74	6.2
H6	PH	female	70	6.4
M1	TX	female	54	5.8
M2	PH	female	45	3.2
M3	TX	female	57	6.3
M4	TX	male	50	3.7
M5	PH	male	66	5.5

The protein content of the PCLS was determined by dissolving the remaining pellet in 5 M NaOH for 30 min. After dilution with water to a concentration of 1 M NaOH, the protein content of the samples was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany) using bovine serum albumin (BSA) for the calibration curve. The ATP content of the slice was expressed as pmol/μg protein.

2.8. Histomorphology of PCLS

Mouse (n=3), rat (n=3) and human (n=6) PCLS, incubated with 2 mM of APAP or AMAP (24h), were subjected to morphological evaluation. PCLS were fixed in 4% buffered formaldehyde in phosphate-buffered saline solution for 24h at 4°C. Thereafter, PCLS were stored in 70% ethanol at 4°C. Embedding in paraffin, sectioning (4 μm) and staining with hematoxylin and eosin (H&E) was performed as described previously [28]. Slice integrity was determined by estimation of the percentage of viable cells by two independent observers who scored the sections blindly.

2.9. Proteinprofiling of PCLS media

Mouse PCLS (n=3) were incubated with APAP (1 mM) or AMAP (3 mM) and rat PCLS (n=3) were incubated with APAP (5 mM) or AMAP (5mM) for 24h. Thereafter, medium samples were collected and snap-frozen in liquid nitrogen and stored in -80°C. Pretreatment of medium samples using Magnetic Beads-based Hydrophobic Interaction Chromatography 8 beads (MB-HIC C8) (BrukerDaltonics, Bremen, Germany), which bind hydrophobic proteins was performed as described previously [29]. An internal standard (synthetic hepcidin-24) (Peptide International Inc., Louisville, KY, USA) was used to enable comparison between samples. A 2 μl volume of the prepared sample was applied to a MSP 96 polished steel MALDI target plate under nitrogen flow, followed by 1 μl of 5 mg/mL α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 0.5% trifluoroacetic acid. Mass-to-charge (m/z) spectra were generated using MALDI-TOF MS (Microflex LT with software flexControl Version 3.0) (BrukerDaltonics, Bremen, Germany) in positive, linear ion mode and 350 laser shots. Initial laser power was 50% for 1-20 kDa measurement, Laser Attenuator; Offset 25% and Range 20%. Pulsed ion extraction was set to 250 ns. Samples were measured in a 1-20 kDa mass range. Calibration was performed using protein calibration standard I (BrukerDaltonics, Bremen, Germany).

2.10. Measurement of metabolites of APAP/AMAP

Metabolites of APAP (0.5 mM) or AMAP (0.5 mM) or DMSO (0.1%) were measured after 24h incubation of rat (n=3), mouse (n=3) and human (n=5) PCLS. PCLS and their corresponding media were collected and sonicated together to disrupt the tissues or cells. 10% of the sample volume of ice-cold 10% $HClO_4$ was added to precipitate the proteins. Samples were then centrifuged for 15 min on 14000 rpm. Supernatants were filtered through membrane filters before they were analyzed. Metabolites of APAP and AMAP were analysed by LC-MS/MS using a Luna 5 μm C18 column (150 mm x 4.6 mm i.d.) (Phenomenex, Torrance, CA, USA) as the stationary phase. A gradient was constructed by mixing the following mobile

phases: solvent A (1% acetonitrile, 99% water, and 0.2% formic acid) and solvent B (99% acetonitrile, 1% water, and 0.2% formic acid). The first 5 min were isocratic at 0% solvent B; from 5 to 30 min, the concentration of solvent B linearly increased to 75%; from 30 to 35 min, there was a linear decrease to 0% B, and it was maintained at 0% for re-equilibration until 40 min. The flow rate was 0.5 mL/min. Samples were injected at an injection volume of 100 μl. Metabolites were identified by mass spectroscopy and quantified by UV/VIS detection at 254 nm. APAP and AMAP were quantified using appropriate standard curves. The extinction coefficients of APAP, AMAP and the metabolites APAP-glucuronide (APAP-gluc), APAP-sulfate (APAP-sulf) and APAP-glutathione (APAP-GSH) were determined by measuring the absorbance in the UV/VIS detector also at 254 nm. In contrast to what was previously reported [30], the extinction coefficient of APAP-GSH (0.00532 $M^{-1}cm^{-1}$) was actually lower than that of APAP itself (0.00837 $M^{-1}cm^{-1}$), whereas those of APAP-gluc (0.00808 $M^{-1}cm^{-1}$), and APAP-sulf (0.00781 $M^{-1}cm^{-1}$) were only slightly lower. As APAP-NAC was not available, the extinction coefficient of APAP-NAC was assumed to be the same as APAP-GSH. The extinction coefficients of all the metabolites of AMAP were assumed to be the same as AMAP as these metabolites were not available. For AMAP-gluc and AMAP-sulf, which appeared to be the main metabolites, this assumption seems reasonable as also for APAP-gluc and APAP-sulf the extinction coefficients were close to that of APAP (0.98 fold and 0.94 fold). An Agilent 1200 Series Rapid resolution LC system was connected to a hybrid quadrupole-time-of-flight (Q-TOF) Agilent 6520 mass spectrometer (Agilent Technologies, Waldbronn, Germany), equipped with electrospray ionization (ESI) source and operating in the positive mode with the MS ion source parameters previously described [31]. Class VP 4.3 software package was used for the determination of peak areas in the UV chromatograms. The MassHunter Workstation Software (version B.02.00) was used for system operation and data collection. Data analysis was performed using Agilent MassHunter Qualitative analysis software.

2.11. Statistics

Each experiment was performed with a minimum of 3 livers (rat and mouse) or 5 livers (human) using PCLS in triplicates from each liver. HepG2 experiments were performed in three experiments with triplicate incubations in each experiment. Results were compared using two-tailed paired Student's t-test (Fig. 1) or one-way ANOVA with a Dunnett post-hoc test (Fig. 3) using GraphPad Prism 5.0 statistics program (GraphPad Software Inc., San Diego, CA, USA). P-value <0.05 was considered statistically significant.

3. Results

3.1 Cytotoxicity of APAP and AMAP in PCLS

Mouse, rat, and human PCLS remained viable in the incubation system during 24h incubation (Figs. 1 & 2). The effect of APAP and AMAP was tested at three different concentrations (1, 3, 5 mM) by comparing the ATP content of the treated PCLS with that of the control PCLS after 24h (Fig. 1). In mouse PCLS, incubation with 1 mM APAP resulted in significant toxicity, while both 3 mM and 5 mM APAP turned the slices' color into black and caused total loss of viability (Fig. 1). Less but significant toxicity was also observed in rat and human PCLS incubated with APAP at 3 mM and 5 mM. Incubation of mouse PCLS with AMAP did not result in any significant toxicity except at the highest dose (5 mM). However, incubation with AMAP resulted in significant toxicity in both rat and human PCLS at all concentrations tested. Surprisingly, AMAP was actually more toxic than APAP at each concentration (Fig. 1).Measurement of LDH leakage in the media of mouse and rat PCLS showed comparable results (data not shown).The approximate TC_{50} values (the concentration of drug that reduces the viability to 50%) for APAP were: 1 mM (mouse), 4.7 mM (rat) and >5 mM (human). The approximate TC_{50} values for AMAP were: 4.3 mM (mouse), 2 mM (rat) and 1 mM (human).

Viability of PCLS was also assessed by histomorphology of mouse, rat and human PCLS incubated with 0.2% DMSO (control), 2 mM APAP or 2 mM AMAP for 24h. The histomorphological scoring for control PCLS was approximately 70% taking into account the damage of the outer cell layers by the slicing. The histomorphology data were largely in accordance with the ATP data. Mouse PCLS incubated with 2 mM APAP showed extensive necrotic areas, while incubation with the same concentration of AMAP showed minor toxicity. On the other hand, incubation with AMAP of both rat and human PCLS resulted in overt toxicity that was equal to or more than the toxicity caused by the same concentration of APAP. There were more necrotic areas in AMAP-treated rat PCLS than in APAP-treated ones, while the treatment with 2 mM of APAP and 2 mM of AMAP of human PCLS resulted in similar levels of necrosis. AMAP-treated PCLS of all 3 species showed signs of karyorhexis (destructive fragmentation of the nuclei of dying cells), which was not observed in the vehicle-treated or APAP-treated PCLS. Based on histomorphological scoring of the PCLS from several individual experiments (Fig. 3), there were more viable cells in AMAP-treated (81%) than APAP-treated (7%) mouse PCLS compared to control incubated PCLS, while the opposite was true for rat PCLS (APAP-treated: 91%, AMAP-treated: 68%). APAP and AMAP induced a similar degree of necrosis in human PCLS (approximate viability: 67%).

3.2. Protein profiling of media from APAP- and AMAP-treated PCLS

The protein profiles in the media following 24h incubation of mouse and rat PCLS with vehicle (DMSO), AMAP (3 mM for mouse and 5 mM for rat) or APAP (1 mM for mouse and 5 mM for rat) were measured (Fig. 4). The protein profile of APAP-treated mouse PCLS were substantially different from those of control PCLS. However, the profile of AMAP-treated mouse PCLS was similar to that of the control PCLS. In rat PCLS, the protein profiles after treatments with APAP or AMAP were similar and clearly different from those of the control PCLS. They resembled the protein profile following APAP treatment in mouse PCLS. This suggests that, in rat PCLS, AMAP and APAP treatment caused a similar effect.

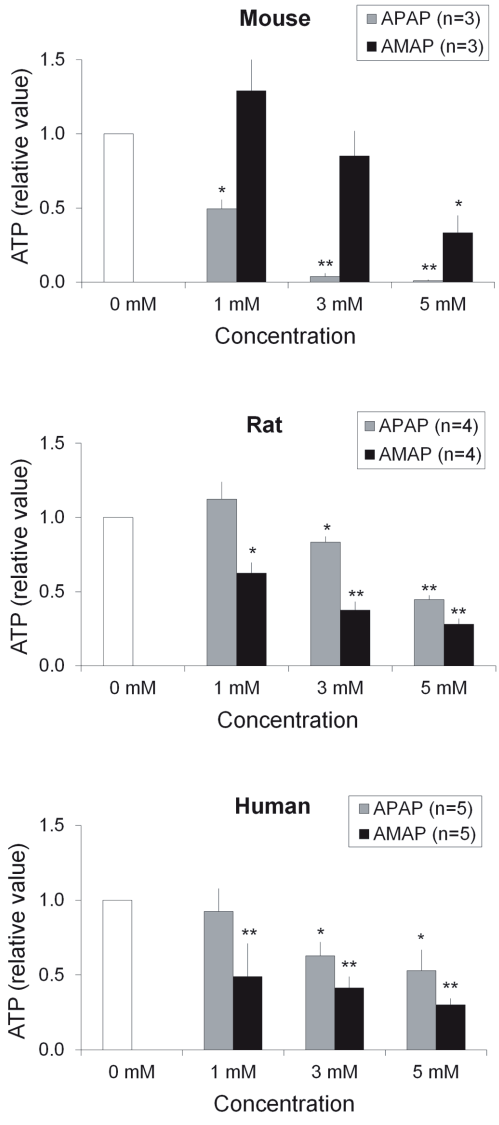

Figure 1. Viability of mouse, rat, and human PCLS, measured as ATP content of PCLS after 24h incubation with different concentrations of APAP (gray bars) or AMAP (black bars). ATP content was expressed as relative values to the corresponding controls: vehicle-treated PCLS (open bars). The absolute ATP content of the control PCLS were: 7.7 (mouse), 13.1 (rat), and 7.8 (human) nmol/mg protein. Data represent the average ± SEM of three (mouse), four (rat) or five (human) individual experiments, using 3 PCLS per experiment. Results are compared with two-tailed paired Student's t-test: *P < 0.05 vs. control, **P<0.005 vs. control.

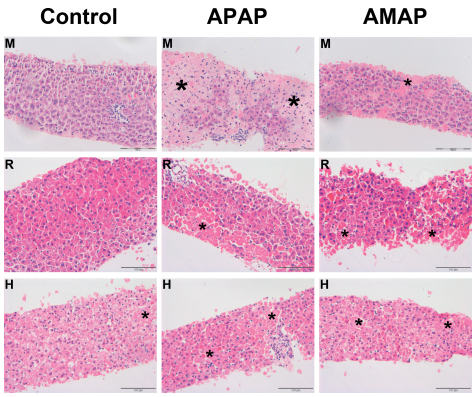

Figure 2. Histomorphology of PCLS treated with vehicle only (control), 2 mM APAP or 2 mM AMAP for 24h. Three individual experiments were performed with mouse (M) and rat (R) PCLS and six individual experiments were performed with human (H) PCLS. A representative picture is shown for each species. The sections were stained by hematoxylin-eosine, bar = 100 µm, magnification = 100x. Some examples of the necrotic areas are indicated by * symbols.

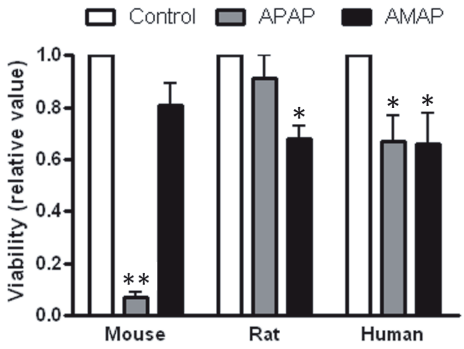

Figure 3. Viability of PCLS from mouse, rat and human treated for 24h with 2 mM APAP (gray bars) or 2 mM AMAP (black bars) based on histomorphological scoring of H&E stained sections. The values were expressed as relative to the corresponding control (vehicle-treated PCLS, open bars) for each species. The absolute values for the controls were 71% (mouse), 70% (rat), and 66% (human). Data represent the average of three (mouse and rat) or six (human) individual experiments ± SEM, using 3 PCLS per experiment. Results are compared with one-way ANOVA with a Dunnett post-hoc test *P < 0.05 vs. control, **P < 0.0001 vs. control.

3.3. Metabolism of APAP- and AMAP-treated PCLS

Metabolites in the media, which were produced after 24h incubation of mouse, rat and human PCLS with 0.5 mM of APAP and AMAP were measured (Fig. 5). The extinction coefficients of all AMAP metabolites were assumed to be the same as of AMAP due to the unavailability of pure AMAP metabolites. Thus, the calculated amounts of metabolites,

especially ones of AMAP, should be considered as an estimation instead of absolute values. The PCLS from all species produced qualitatively the same metabolites of APAP and AMAP; however, quantitative differences were observed both in the amount as well in the profile. The total amount metabolized during 24 hours was 56%, 45% and 26% for APAP and 85%, 67%, 60% for AMAP in mouse, rat and human PCLS respectively. Both glucuronide (APAP-gluc and AMAP-gluc) and sulfate (APAP-sulf and AMAP-sulf) metabolites were detected as the main metabolites in APAP- and AMAP-treated PCLS. Only in APAP-treated PCLS a glutathione-conjugate (APAP-GSH) and N-acetylcysteine-conjugate (APAP-NAC), which is the breakdown product of APAP-GSH [32], could be detected. In AMAP-treated PCLS, three hydroquinone metabolites were detected, namely 3-hydroxy-acetaminophen (3-OH-APAP); 2,5-dihydroxy-acetanilide (2,5-diOH-AA) and 3-methoxy-acetaminophen (3-OMe-APAP).

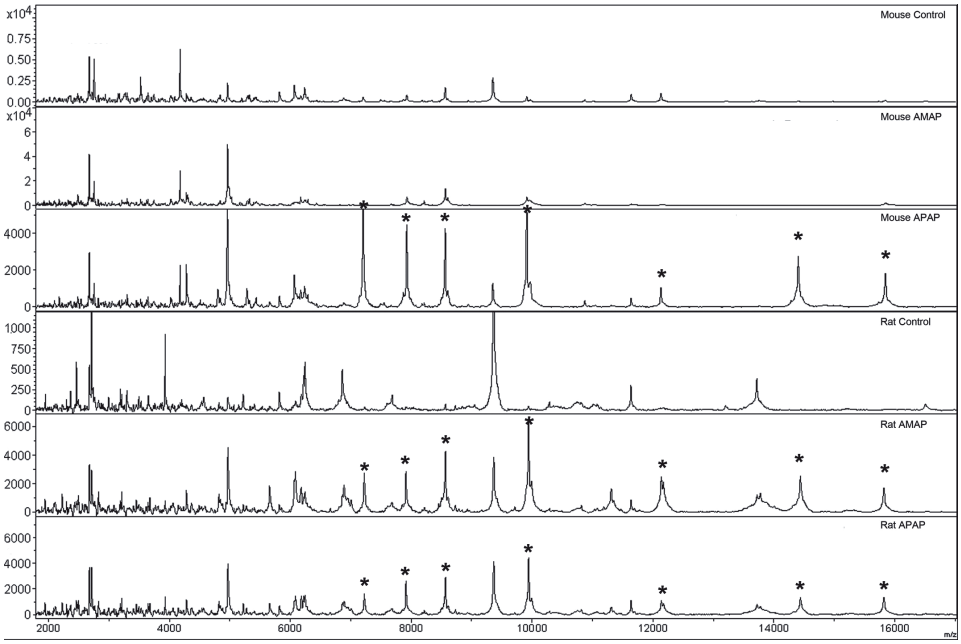

Figure 4. Representative protein profiles, which were measured by MALDI-TOF MS, of media of PCLS from mouse and rat following 24h APAP and AMAP treatment. For each profile, the Y-axis was adjusted so that the peak corresponding to the internal standard (IS, hepcidin 24; 2673.9 Da) was at 50% of the Y-axis to allow comparison of relative protein peak intensity between spectra. The * symbols indicate some of the protein peaks that are present in medium of APAP-treated rat and mouse PCLS and AMAP–treated rat PCLS, but are low or lacking in medium of control slices or mouse PCLS treated with AMAP.

In both mouse and human PCLS, APAP was mainly metabolized into APAP-gluc, followed by APAP-sulf, while the rat PCLS produced more APAP-sulf than APAP-gluc. Both APAP-GSH and APAP-NAC were detected albeit in low quantities (at 24h <10 nmol) in all species except in mouse where the amount of APAP-GSH produced (~65 nmol) was similar to APAP-sulf. In PCLS from all three species, the two main metabolites of AMAP, AMAP-gluc and AMAP-sulf were produced in higher amounts than the equivalent metabolites of APAP. The amount of APAP-gluc produced in mouse was twice the amount produced in human and three times the amount produced in rat, whereas AMAP-gluc was produced more in human than in the other two species. Rat produced much more APAP-sulf than mouse and human, while mouse produced the most AMAP-sulf, followed by the rat and then human. Interestingly, PCLS of the mouse, the most sensitive species for APAP toxicity, produced approximately ten times more APAP-GSH than rat PCLS and almost thirty times more than human PCLS. APAP-NAC was produced in small amounts in all species with the order of mouse>human>rat. In AMAP-treated PCLS, catechol and methylated catechol metabolites were produced in low amounts but surprisingly, in mouse PCLS they were produced in much higher amounts than in rat or human PCLS.

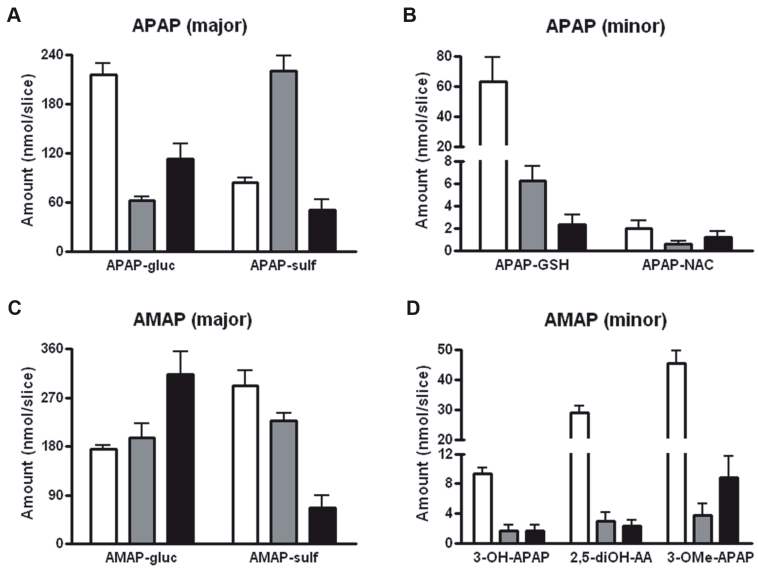

Figure 5. Metabolites that were produced by PCLS during 24h incubation with APAP or AMAP (0.5 mM, total amount = 650 µmol). The graphs show all major (A and C) and minor metabolites (B and D) of APAP (A and B) and AMAP (C and D) from mouse (open bars), rat (gray bars) or human (black bars). Data were the average of three (mouse and rat) or five (human) individual experiments.

4. Discussion

AMAP, a regioisomer of APAP with similar analgesic and anti-pyretic properties has been frequently used as a non-toxic analog in studies on the mechanism of APAP-induced liver injury. APAP is probably the most extensively investigated drug but still its mechanism of toxicity is not fully elucidated. The concentration range (1-5 mM) used in this study is physiologically relevant because similar concentrations have been reported in plasma of APAP overdose patients [33]. Our results indicate that mouse (TC_{50}≈1 mM) is the most sensitive species to APAP toxicity, followed by rat (TC_{50}≈4.7 mM) and human (TC_{50}>5 mM) (Fig. 1). The order of the species sensitivity to APAP is in line with that reported for primary hepatocytes with TC_{50} values of 3.8 mM (mouse), 7.6 mM (rat), and 28.2 mM (human) [34]. In a recent study using the human cell line HepaRG[35] and HepG2 cells (Herpers, unpublished data), a much higher APAP concentration (5-20 mM) was needed to observe toxicity. It can be concluded that species differences are similar in hepatocytes and PCLS, but PCLS appear more sensitive to APAP-induced toxicity. This may suggest the possible involvement of non-parenchymal liver cells in APAP-induced hepatotoxicity. Kupffer cells are thought to exacerbate APAP-induced hepatotoxicity by increasing the synthesis of oxygen free radicals [36], although this finding was not confirmed by Ju*et al.*, who found that Kupffer cells may have a protective role in addition to its pro-toxicant role in drug-induced acute liver injury [37]. Moreover, they are involved in the regulation of phase II metabolism in the adjacent hepatocytes [38].

In contrast to APAP toxicity observed at all concentrations tested (1-5 mM), AMAP was toxic only at 5 mM in mouse PCLS, exhibiting a TC_{50} value of ca. 4.3 mM (Fig. 1). Similar findings were observed in mouse TAMH cells and in isolated mitochondria, glutathione depletion was more severe with APAP than AMAP [15]. So far, the toxicity of AMAP has not been reported for rat or human and the mechanism of AMAP toxicity was virtually unknown. Surprisingly, AMAP caused significant cytotoxicity even at 1 mM in rat (TC_{50} = 2 mM) and human (TC_{50} = 1 mM) PCLS and appeared more toxic than APAP at all tested concentrations in rat and human PCLS (Figs. 1,2,3).

To confirm the observed effects of AMAP, we assessed the profiles of proteins released by the PCLS in the medium. We found similar protein profiles after APAP treatment in mouse PCLS and after both APAP and AMAP treatments in rat PCLS, which differed from the corresponding control PCLS or the profile after AMAP treatment in mouse PCLS (Fig. 4). The proteins released by mouse and rat PCLS after APAP- or AMAP-induced toxicity appear to be related to the resulting liver injury and could potentially represent new toxicity biomarkers.

In order to obtain more insight into the species difference in AMAP and APAP toxicity,

we investigated the metabolites produced by mouse, rat and human PCLS. In mouse and human PCLS, the main biotransformation pathway for APAP was glucuronidation, whereas sulfation was responsible for 76% of all metabolites produced in rat PCLS. The relative amount of APAP-gluc (~60% of the total metabolites formed) and APAP-sulf (~30% of the total metabolites) produced in human PCLS (Fig. 5A) is in line with the observations *in vivo*[39]. Among the three species, mouse produced not only the highest amounts of phase II metabolites (APAP-gluc and APAP-sulf), but also the amount of APAP-GSH produced was approximately 10 and 30 times higher than that in rat and human respectively (Fig. 5C). This suggests that the rate of formation of the reactive metabolite NAPQI by phase I metabolism is also higher in mouse which may explain the higher toxicity found.

In both mouse and rat PCLS, sulfation was the main metabolic pathway of AMAP (Fig. 5B). Conversely, the estimated amount of AMAP-gluc produced in human PCLS was almost 5 times higher than AMAP-sulf showing a difference in AMAP detoxification pathways between men and animals. In contrast to what might be expected based on the toxicity data, AMAP oxidative metabolites comprise approximately 20% of all metabolites produced in mouse PCLS, while the percentage is much lower in rat and human (3% and 5% respectively). However, no GSH-conjugate of AMAP was found in any species, even when a higher concentration of AMAP (2.5 mM) was utilized (data not shown). AMAP is reported to be more susceptible to oxidative metabolism than APAP, but in contrast to APAP, AMAP cannot be directly oxidized to a reactive quinone imine [11]. In our study, AMAP was metabolized to three different proximate reactive hydroquinone metabolites; 3-OH-APAP, 2,5-diOH-AA and 3-OMe-APAP, in line with previously reported data [40]. These metabolites can in principle be converted to the reactive quinone metabolites, AMAP-o-benzoquinone (AMAP-o-Q), AMAP-p-benzoquinone (AMAP-p-Q) and 3-methoxy-acetyl-p-benzoquinone-imine (MAPQI) respectively. The hydroquinone metabolites can be subjected to further glucuronidation or sulfation. The reactive quinone metabolites of AMAP can be conjugated with GSH [9], but not as extensively as NAPQI and this was shown to occur only at very high doses of AMAP [10, 11]. Despite the detection of 5-10 times higher estimated amounts of hydroquinone metabolites in mouse PCLS compared to human and rat (Fig. 5D), mouse was the least sensitive species to AMAP toxicity.One possible explanation may be that the conversion of the hydroquinone to the ultimate quinone metabolites may occur at a higher rate in human and rat liver but these may remain undetected as the metabolites rapidly bind covalently to cell components, thereby inducing toxicity. Alternatively, quinone metabolites might be more efficiently reduced by NAPDH quinoneoxidoreductase1 in mice, which might explain the inverse relationship between the level of these proximate reactive metabolites and AMAP-induced toxicity.

Administration of APAP and AMAP *in vivo* resulted in similar levels of covalent binding to proteins in mice [13] and hamsters [10]. Reactive AMAP metabolites were bound more extensively to microsomal and cytosolic proteins, whereas reactive metabolites of APAP were bound more extensively to mitochondrial proteins in mouse liver [8]. Additionally, while a large number of APAP adducts were detectable in all hepatic fractions [41], the majority of AMAP adducts were found in the endoplasmic reticulum (microsomes). A major AMAP adduct was observed at 50 kDa in mouse [13], which was later identified as CYP2E1 [42]. Therefore, AMAP, but not APAP, has the ability to function as a mechanism-based inhibitor of CYP2E1, which may explain the low toxicity of AMAP in mice. Whether this inhibitory effect is stronger in mouse CYP2E1 than in rat or human CYP2E1 is unknown. Our results showed that more than 85% of AMAP was metabolized in mouse PCLS, while the percentage in rat and human were only 67% and 60% respectively, resulting in a higher exposure to AMAP in the human and rat PCLS. Moreover, co-incubation of AMAP and 1-aminobenzotriazole, a non-selective mechanism-based inactivator of cytochrome P450s in both rat and human [43, 44] that was shown not to inhibit UDP-glucuronosyltransferase and sulfotransferase activities [45], did not improve the viability of AMAP-treated rat and human PCLS (unpublished data). Therefore, it cannot be excluded that AMAP itself is cytotoxic to the liver.

In conclusion, there are pronounced species differences in toxicity and metabolism of APAP and AMAP. This is the first report on the toxicity of AMAP in human and rat tissue, and we have shown that AMAP is at least as toxic as or even more toxic than APAP *ex vivo* in rat and human PCLS. Consequently, AMAP can no longer be considered as a general non-toxic isomer of APAP. The concentration of APAP causing toxicity in the human PCLS is lower than that previously found by others using HepG2 cells, HepaRG cells and also primary hepatocytes, which are considered as the gold standard of in vitro toxicity testing, possibly due to the high metabolic competence and the presence of non-parenchymal cells in the PCLS. Finally, the observed species differences in the toxicity of AMAP in mouse and human emphasize the importance of using human tissue in safety assessment. Therefore, the use of human PCLS may contribute not only to the reduction in the use of experimental animals but also to a better prediction of human hepatotoxicity.

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3

Proteomic profiling in medium from mouse, rat and human precision-cut liver slices for biomarker detection regarding drug-induced liver injury

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Abstract

Drug-induced liver injury (DILI) is among the leading causes for drug withdrawal. Currently, there is a lack of predictive biomarkers. In this study, precision-cut liver slices (PCLS) were used to identify biomarkers for DILI from proteins excreted by the liver. PCLS from mouse, rat and human origin were incubated with acetaminophen (APAP), 3-acetamidophenol (AMAP), diclofenac (DF) and lipopolysaccharide (LPS) for 24-48 h. Profiling of proteins in the medium of PCLS from all species treated with APAP using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry demonstrated the same proteins in the medium as in urine of mice after APAP treatment. Similar profiles were obtained in human and rat PCLS treated with APAP and AMAP, and in mouse PCLS treated with APAP or DF, but not with AMAP, which is in line with our findings that AMAP is toxic in human and rat but not in mouse PCLS. LPS treatment did not result in a profile different from control. Further analysis showed that the concentration of hepcidin, an iron-regulating hormone, was reduced in PCLS medium after APAP, AMAP or DF treatment, but increased levels were found after LPS exposure. In mouse but not in human PCLS, superoxide dismutase (SOD) 1 was induced slightly by LPS and to a larger extent by DF, whereas in rat PCLS, SOD1 was induced by DF only. In conclusion, the PCLS system is a promising, translational model for studying DILI and for identification of species differences and potential biomarkers.

1. Introduction

The most common adverse drug reaction leading to drug withdrawal is drug-induced liver injury (DILI) [1]. The incidence for DILI has been estimated to be at least 10-15 cases per 100,000 patient years [2]. Despite the efforts to study DILI, for most drugs the underlying mechanisms have not been fully elucidated yet. In addition, particular drugs can cause a rare and severe form of DILI, without a straightforward dose-effect relationship or a relation with their therapeutic mode of action. These adverse reactions are, therefore, described as idiosyncratic. Currently, there are no adequate biomarkers to detect idiosyncratic DILI in patients, in preclinical animal studies or in *in vitro*models during drug development [3]. Because of this, identification of novel biomarkers for DILI is difficult and new methods to address this issue are being explored [4, 5]. Although for non-idiosyncratic DILI more knowledge on potential mechanisms is available compared to idiosyncratic DILI, the prediction from preclinical data is limited and better preclinical prediction models with their accompanying biomarkers are needed.

Precision-cut liver slices (PCLS) of mouse, rat and human liver are being increasingly used to study the hepatotoxic effects of many compounds [5-9]. The advantage of this *ex vivo* model over *in vitro* models, including cell cultures, is that the structure of the liver tissue is maintained in PCLS as well as the presence and interactions of all parenchymal and non-parenchymal cell types, including cell-matrix interaction [10, 11]. Moreover, PCLS retain the expression and activity of phase I and phase II metabolizing enzymes well, the function of which is comparable to the *in vivo* situation [7, 12-15]. Furthermore, it has been shown that rat PCLS demonstrate the same responses to toxic compounds as observed *in vivo* based on microarray data [6].

Recently, we have used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to assess and identify potential novel biomarkers for DILI [16]. For this, mice were administered a single dose of acetaminophen (APAP) as a model compound for hepatocellular liver injury and 24 h urine samples were collected. From the urine samples, multiple proteins could be identified that were related to APAP-induced liver injury. Although the data suggested that these candidate urinary biomarkers originate from the liver, this is difficult to prove in an *in vivo* experimental setup. Here, we tested whether the PCLS system could be used to demonstrate that the proteins we found in urine indeed originated from the liver. Furthermore, we aimed to identify protein biomarkers that could be used as early and sensitive organ-specific biomarkers, which could be applied in human urine samples during clinical studies. To this end, we investigated the protein profiles in the PCLS medium after exposure to 3-acetamidophenol (AMAP) and diclofenac (DF) in addition to APAP. AMAP was shown previously to induce species-specific toxicity in PCLS of human and rat, but not of mouse[8]. Diclofenac (DF) is a non-steroidal anti-

inflammatory drug that has been known to cause idiosyncratic DILI [17] and to induce acute hepatotoxicity similar to APAP [18].

2. Methods

2.1. Animals for PCLS studies

Female C57BL/6 mice weighing 20-24 g and male Wistar rats (HsdCpb:WU) weighing 300-350 g were obtained from Harlan (Horst, the Netherlands). The mice and rats were housed on a 12-h light/dark cycle in a temperature- and humidity-controlled room with food (Harlan chow no 2018, Horst, the Netherlands) and tap water *ad libitum*. The animals were allowed to acclimatize for at least seven days before experimentation. The experimental protocols were approved by the Animal Ethical Committee of the University of Groningen. Under isofluorane/O_2 anesthesia, the liver was excised and placed into ice-cold University of Wisconsin (UW) organ preservation solution (DuPont Critical Care, Waukegab, IL, USA).

2.2. Human liver tissue

Pieces of human liver tissue were obtained from patients undergoing partial hepatectomy (PH) for the removal of carcinoma or from liver tissue remaining as surgical waste after split liver transplantation (TX), as described previously [7, 11]. The experimental protocols were approved by the Medical Ethical Committee of the University Medical Center Groningen.

2.3. Preparation of the PCLS

PCLS were made as described previously [5, 8, 19]. In brief, cylindrical liver cores were made using a 5mm biopsy punch (Kai Industries, Seki, Japan). These cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs-Henseleit buffer saturated with carbogen (95% O_2 and 5% CO_2). PCLS (5 mm diameter, 200-300 μm thick and ca. 4.5-5.5 mg wet weight) were stored in ice-cold UW solution until incubation.

2.4. Incubation of the PCLS

Incubation of PCLS in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria) was performed as described before [19]. In brief, PCLS were pre-incubated at 37°C for 1 h individually in 1.3 ml Williams' medium E with glutamax-1 (Gibco, Paisley, UK), supplemented with 25 mM D-glucose and 50 μg/ml gentamicin (Gibco, Paisley, UK) (WEGG medium) in a 12-well plate with shaking (90 times/min) under saturated carbogen atmosphere. Pre-incubation allows the PCLS to restore their ATP levels. After pre-incubation, PCLS were transferred to fresh WEGG medium and incubated with vehicle

(final concentration of DMSO during incubation ≤0.5%), APAP, AMAP, DF or LPS further for 24h. The human PCLS were incubated with APAP or AMAP for 48 h instead of 24 h, as no changes were observed after 24 h in protein profiles compared to control slices. The concentration of drugs used were based on previous dose-response studies and aimed to induce low to moderate damage to the slices, and were 1 mM APAP and 3 mM AMAP for mouse, 5 mM APAP and AMAP for rat and 3 mM APAP and AMAP for human PCLS. LPS was used in a concentration of 20,000 EU/mL for all species. The concentration of DF was 200 μM in mouse, 350 μM in rat and 500 μM in human PCLS. The medium was collected, snap frozen in liquid nitrogen and stored at -80 °C until further use.

2.5. ATP content of PCLS

Viability of PCLS was determined after incubation by measuring the ATP content of the PCLS according to the method described earlier [19]. In brief, at the end of incubation, three replicate PCLS were collected individually in 1 ml 70% ethanol (v/v) containing 2 mM EDTA (pH 10.9) and snap-frozen in liquid nitrogen and stored at -80°C until analysis. The samples were homogenized using a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA) and centrifuged for 3 minutes at 13000 rpm and 4°C. The supernatant was diluted 10 times with 0.1 M TrisHCl containing 2 mM EDTA (pH 7.8) to reduce the ethanol concentration. The ATP content of the supernatant was measured using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) in a black 96-well plate Lucy1 luminometer (Anthos, Durham, NC, USA) using a standard ATP calibration curve.

The protein content of the PCLS was determined by dissolving the remaining pellet in 200 μl of 5 M NaOH for 30 min. After dilution with water to a concentration of 1 M NaOH, the protein content of the samples was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany) using bovine serum albumin (BSA) for the calibration curve.

2.6. Mouse urine samples

For validation of the PCLS model, urine samples obtained from a previously performed mouse study [16] were used. In short, male FVB mice were treated with a single i.p. dose of 0-350 mg/kg APAP and placed in a metabolic cage for 24 h to collect urine. After 24 h, liver tissue was collected and homogenized using a Mikrodismembrator U (Sartorius Stedim, Nieuwegein, the Netherlands).

2.7. Protein profiling

Proteins were isolated from urine, liver homogenates or PCLS medium using Magnetic Beads based Hydrophobic Interaction Chromatography 8 beads (C8; BrukerDaltonics

GmbH, Bremen, Germany) that bind hydrophobic proteins [20]. Synthetic hepcidin-24 (Peptide International Inc., Louisville, KY, USA) was used as internal standard (IS) to enable comparison between samples. For protein profiling, MALDI-TOF MS (Microflex LT with software flexControl Version 3.0, BrukerDaltonics) was used. Of the prepared sample, 1 µl was applied to a MSP 96 polished steel MALDI target plate under nitrogen flow, followed by 1 µl of energy absorbing matrix, 5 mg α-Cyano-4-hydroxy-cinnamic acid (CHCA) in 1 ml 50% acetonitrile (ACN) and 0.5% trifluoro acetic acid (TFA). Mass-to-charge (m/z) spectra were generated using MALDI-TOF MS in positive, linear ion mode and 350 laser shots. Initial laser power; 50% for 1-20 kDa and 60% for 10-160 kDa measurements, Laser Attenuator; Offset 25% and Range 20%. Pulsed ion extraction was set to 250 ns. Samples were measured in the 1-20 kDa mass range and 10-160 kDa mass range. Calibration was performed using protein calibration standard I for 1-20 kDa measurements and protein calibration standard II (both BrukerDaltonics) for 10-160 kDa measurements. Spectra were analyzed by means of the ClinProTools software (Bruker). Relative peak intensities were calculated by dividing mass peak intensity by the peak intensity of the IS.

2.8. Protein identification

Protein identification was performed at the Nijmegen Proteomics Facility (Nijmegen , the Netherlands) using an electrospray ionization mass spectrometer (ESI LTQ; Thermo Fisher Scientific) with a liquid chromatography column placed in front of the ESI probe. Peptide and protein identifications were extracted from the ESI data by means of the search program Mascot using a musmusculus RefSeq36 database. Finally, the peptides and proteins found by Mascot were validated with the in-house designed script PROTON, as described elsewhere [21].

2.9. Hepcidin determination

The concentration of hepcidin in PCLS medium was determined using MALDI-TOF MS analysis as previously described [22].

2.10. Statistical analysis

All experiments were performed in 3 slices of 5 (mouse and human) or 4 (rat) individual livers. Statistics were performed using GraphPad Prism 5.02 (La Jolla, USA), unless indicated otherwise. A p-value of less than 0.05 was considered statistically significant. Data were compared among groups using one-way ANOVA with a Dunnett's post hoc multiple comparisons test.

3. Results

3.1. Species difference in drug sensitivity

The effect of the drugs on PCLS viability was determined by measuring ATP levels after incubation with the different drugs. For APAP and AMAP, previous studies have shown that with the concentrations used in this study, APAP significantly decreased ATP levels in mouse, rat and human PCLS, whereas AMAP treatment did not change ATP levels in mouse PCLS, but significantly reduced ATP levels in rat and human tissue. [8]. This indicated that AMAP was just as toxic as APAP in human PCLS and even more toxic than APAP in rat PCLS. In figure 1, the effe cts of DF and LPS treatment on ATP levels in mouse, rat and human PCLS are demonstrated. Whereas DF reduced the ATP content to 75%, this effect was not significant. Also LPS treatment had no significant effect on ATP levels in mouse PCLS.

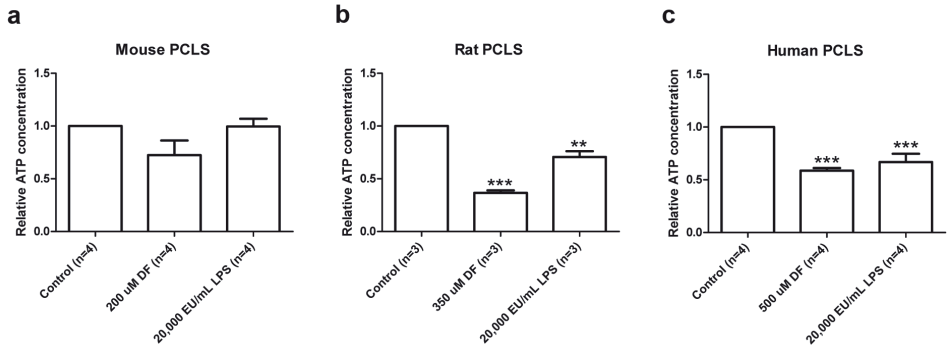

Figure 1. PCLS viability after treatment with DF and LPS

Effect of 24 h incubation of DF and LPS on the ATP content of PCLS prepared from mouse (a), rat (b) and human (c). ** p< 0.01 *** p < 0.001 compared to control.

3.2. Protein profiles of PCLS medium resemble in vivo mouse urinary profiles of APAP-induced liver injury

Previously, we have identified a specific protein profile of mouse urine that correlated with APAP-induced liver injury [16]. By profiling the proteins of mouse liver homogenates, we now demonstrate that the proteins observed in urine of mice with APAP-induced liver injury could originate from the liver (Figure 2). However, there was no difference in the protein profiles of liver homogenates obtained from control mice and mice with APAP-induced liver injury. After profiling mouse PCLS medium samples, the same protein peaks found in urine of mice with APAP-induced liver injury, were found in medium of mouse liver slices upon incubation with APAP [8]. Protein identification

revealed that the same key proteins that differentiated between control and APAP-induced liver injury in mouse urine samples, differed between control and APAP-treated PCLS medium (Table 1).

3.3. Similar toxicity profiles in mouse, rat and human PCLS after APAP treatment

We profiled mouse and rat PCLS medium samples incubated with APAP or AMAP for 24 h and human PCLS medium incubated with APAP or AMAP for 48 h. We confirmed the data previously presented by Hadi et al 2012b, as we demonstrated that APAP and AMAP caused a similar toxicity profile in rat PCLS as APAP in mouse PCLS, whereas AMAP treatment in mouse PCLS did not lead to a toxicity protein profile (Figure 3A). In addition, we showed that both APAP and AMAP treatment in human PCLS led to a toxicity profile similar to APAP treatment in mouse PCLS. It thus appears that, besides rat PCLS, AMAP is also toxic in human PCLS as opposed to mouse PCLS.

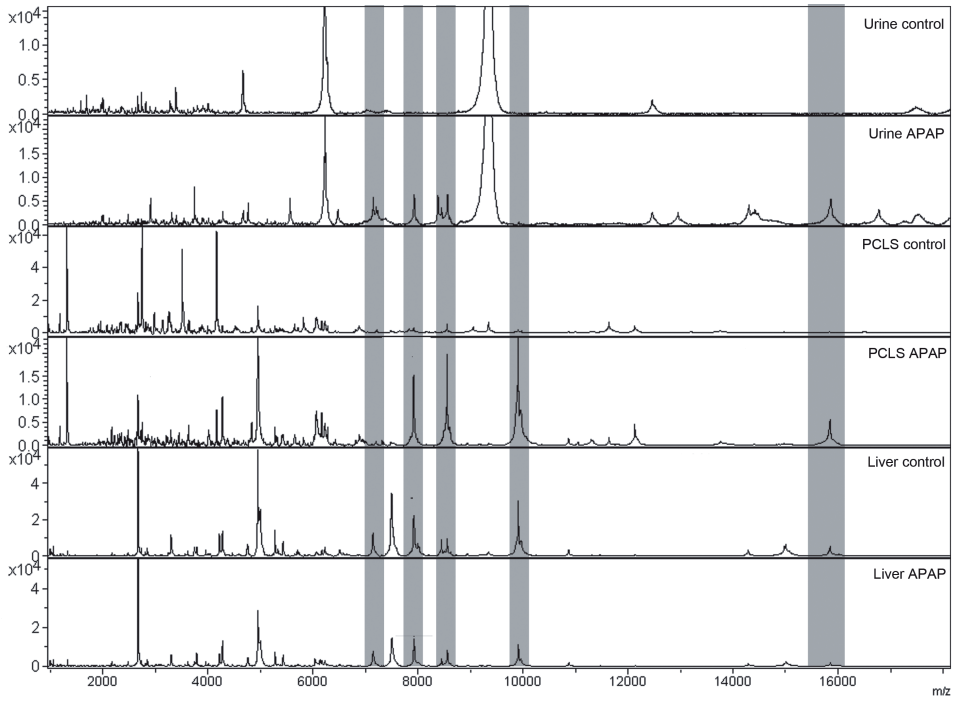

Figure 2. Protein profiles of mouse urine and liver homogenate, and mouse PCLS medium after APAP treatment

Protein profiles of protein masses (m/z in Da) versus peak intensity (arbitrary units) of mouse urine, PCLS medium and liver homogenate of control and APAP treatment. Shaded areas indicate proteins that differ between control and APAP treatment.

One potential marker associated with DILI is hepcidin, for which it was demonstrate that hepatic and plasma levels were down-regulated in mice with APAP-induced liver injury as a result of oxidative stress [23]. To investigate whether acute APAP-induced hepatotoxicity in PCLS is mediated by a similar mechanism as observed in vivo, we measured the concentration of the iron-regulating hormone hepcidin in PCLS medium. Lipopolysaccharide (LPS) was used as a positive control in these experiments to assess the physiological response of hepatic hepcidin regulation in the PCLS, as LPS is a strong inducer of hepcidin[22].In concordance with the mouse in vivo data [23], hepcidin concentration was also decreased in PCLS medium of all species after APAP treatment (Figure 3B). AMAP treatment did not affect hepcidin concentration in mouse PCLS medium, but hepcidin concentration was decreased in rat and human PCLS after AMAP treatment. Hepcidin concentrations are known to vary substantially between individual patients [24], which hampers the assessment of statistical significant changes. To demonstrate that the effects of APAP and AMAP on hepcidin in PCLS of all individual donors were similar, we included line graphs that show the hepcidin concentration for all treatments in PCLS obtained in each mouse, rat or human liver slice (Figure 3C).

3.4. DF treatment resembles the toxicity profile of APAP

Incubation of mouse PCLS with DF resulted in protein profiles of PCLS medium that resembled the toxicity profile of APAP, albeit with lower peak intensities (Figure 4A). LPS had minimal effects on the protein profiles. The medium samples of rat and human PCLS treated with DF proved to be difficult to profile using MALDI-TOF MS and as a result the protein profiles were of insufficient quality, as they showed high background signals and low peak intensities. Therefore, we could not compare the protein profiles of rat and human PCLS medium samples after DF incubation with those of mouse PCLS. However, we were able to compare the concentration of superoxide dismutase (SOD) 1 at 15.9 kDa, one of the protein masses identified in the urine of mice with APAP-induced liver injury (Figure 2) that was also present in medium samples of DF-treated mouse PCLS (Figure 3A). SOD1 was absent in control mouse PCLS medium samples, slightly induced after LPS incubation, and greatly induced after DF treatment (Figure 4B). Similarly, SOD1 was absent in control rat PCLS medium samples and was induced by DF, however, not by LPS. In the human PCLS medium samples, SOD1 could already be detected in control samples, whereas levels did not increase after LPS or DF.

In addition, hepcidin concentrations in PCLS medium samples were determined (Figure 5A). Similar to the findings in mouse plasma and PCLS medium after APAP-induced hepatotoxicity, incubation of PCLS with DF resulted in decreased hepcidin concentrations

in the medium samples of mouse, rat and human PCLS. The effect of DF was not statistically significant in rat and human PCLS medium samples due to the high variation, but the line graphs demonstrate that DF treatment decreased the hepcidin concentrations in each sample (Figure 5B). Incubation with LPS induced the hepcidin concentration in all species, which is in agreement with a previously described effect of the endotoxin [22]. These findings confirmed that the PCLS have maintained their physiological regulation of hepcidin.

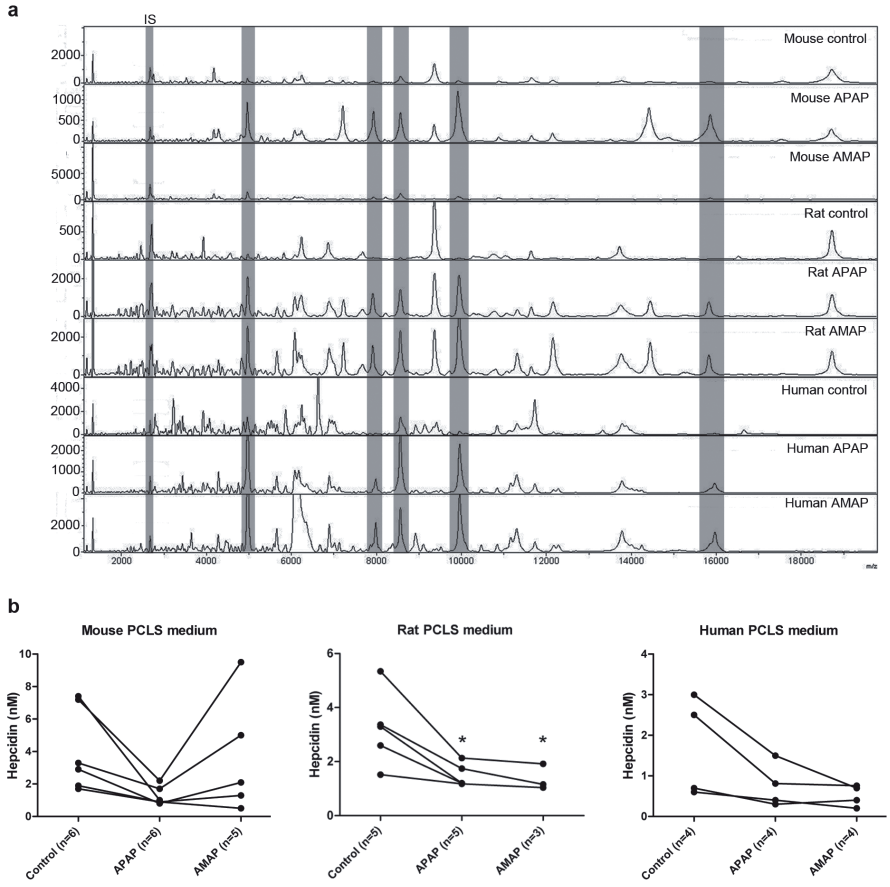

Figure 3. Protein profiles and hepcidin concentration of mouse, rat and human PCLS medium after APAP orAMAP treatment.

Protein profiles of protein masses (m/z in Da) versus peak intensity (arbitrary units) of medium from mouse, rat and human PCLS of control, APAP or AMAP treatment (a). Shaded areas indicate proteins that differ between control and APAP/AMAP treatments. Hepcidin concentrations in medium of control PCLS and after APAP or AMAP treatment for all species (b). Every line represents the hepcidin concentrations for a single PCLS in control situation and after APAP and AMAP treatment. * $p < 0.05$ compared to control.

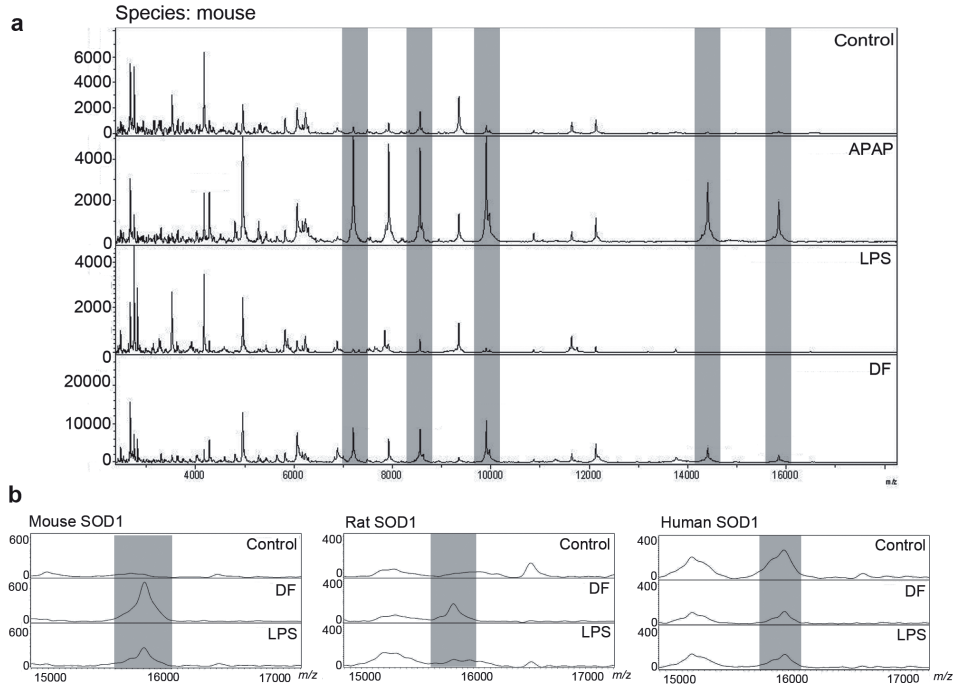

Figure 4. Comparison of PCLS medium protein profiles after incubation of APAP, DF orLPS

Profiles of protein masses (m/z in Da) versus peak intensities (arbitrary units) of medium from mouse PCLS of control and treatment with APAP, LPS and DF (a). Shaded areas indicate proteins that differ between control and APAP or DF treatment. For rat and human PCLS the peak intensity of SOD1 at 15.9 kDa in medium after DF and LPS treatment was compared to SOD1 peak intensity in medium of mouse PCLS (b). Shaded areas here show the SOD1 mass peak.

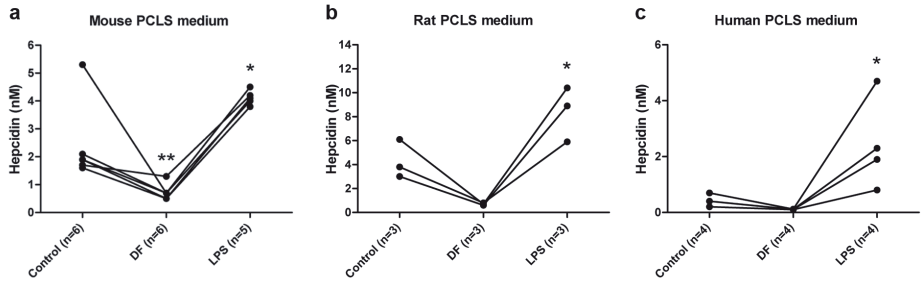

Figure 5.Hepcidinconcentration in PCLS medium after DF or LPS treatment

Hepcidin concentration in medium of mouse (a), rat (b) and human (c) PCLS after treatment with DF or LPS.* p< 0.05; ** p < 0.01 compared to control.

4. Discussion

DILI remains a major problem in drug development, but also is a dangerous complication of drug treatment in patients. Because of species differences and the lack of mechanistic insight for many drugs, identification of biomarkers predictive for DILI is difficult. This study demonstrates the potential of PCLS to identify biomarkers for DILI in a translational approach using model compounds, such as APAP and DF.

Using the PCLS system, we confirmed that the proteins observed previously in 24 h urine samples in response to APAP-induced liver injury [16], including fatty acid binding protein, carbonic anhydrase 3, SOD1 and regucalcin, are released by the liver tissue upon APAP treatment, as they were found in the medium surrounding the PCLS (Table 1 and Fig 2). These proteins all play a role in processes known to be involved or affected in acute liver injury, such as oxidative stress, disruption of calcium homeostasis and mitochondrial dysfunction [25-30]. There were no differences between the protein profiles of liver homogenates of control mice and mice with APAP-induced liver injury. This indicates that the proteins found in PCLS medium and mouse urines with APAP-induced liver injury are normally present in liver tissue and are not produced upon injury. Furthermore, in the *in vivo* study we could not find any signs of kidney injury as a result of APAP administration, which could potentially have influenced the urinary protein composition. We thus found strong support for the hypothesis that with APAP-induced injury, proteins are released by liver tissue and excreted into the urine via the blood.

An advantage of using the PCLS system compared to assessment of biomarkers in urine is that there is less hindrance caused by major urinary proteins, which are abundantly present in rodent urine. The PCLS medium lacks the presence of such proteins and, hence, additional potential biomarkers might be easier to detect. Moreover, because PCLS can be made from organs of multiple species, the system has potential for translational research and can detect species differences in responses to toxic drugs. We analyzed if similar toxicity profiles as caused by APAP in mouse PCLS could also be observed in rat and human PCLS. Not only did APAP treatment result in similar protein profiles in all species, for AMAP toxicity-related protein profiles were found comparable to APAP in rat and human PCLS. Therefore, this study confirmed the species differences with respect to toxicity of AMAP, which is often incorrectly designated as the non-toxic isomer of APAP [8, 31].

PCLS of mouse, rat and human origin were incubated with APAP, AMAP and DF. The doses of these compounds were carefully selected from initial dose-response studies based on the criteria that each drug concentration elicits minimal hepatotoxicity and is within a physiologically relevant concentration limit of 50-times its maximum plasma concentration (C_{max}) value [32]. For LPS, a dose was selected that was predicted to be of minimal toxicity,

but would elicit an inflammatory response by the release of cytokines (Hadi et al 2012b). Accordingly, LPS treatment had minimal effects on ATP levels and protein profiles in the medium compared to the other compounds. In general, the changes in ATP levels corresponded to a large extent with the protein profiles in all species studied. APAP induced a toxicity-related protein profile for all species and decreased the ATP levels. AMAP did not have any effects on ATP level and protein profile in mouse PCLS, whereas it resembled the toxicity pattern of APAP in rat and human PCLS. Although the dose of 200 μM DF did not affect ATP levels in mouse PCLS in these experiments, the protein profile resembled that of APAP, indicating a hepatotoxic effect. It appears that the protein profiles of PCLS medium are a more sensitive read-out of toxicity than ATP levels. Moreover, just slightly higher doses of DF in mouse PCLS decreased ATP levels dramatically (data not shown). In addition, DF treatment significantly decreased ATP levels in rat and human PCLS.

Profiling of rat and human PCLS medium samples after DF treatment proved to be difficult. For some unknown reason, the protein profiles of these PCLS medium samples demonstrated high background signals and generally low peak intensities. Nevertheless, the presence of SOD1 could be confirmed from these spectra and levels were increased in rat PCLS treated with DF. In human PCLS, however, SOD1 was already present in control samples. Human PCLS are prepared from tissue that is more extensively handled before slicing compared to rodent PCLS, as they originate from human left-over tissue from surgery. The presence of SOD1 in control human PCLS indicates that the tissue already showed a stress response and that incubation of DF for 24 h did not have an additional effect on the peak intensity of this protein. Moreover, human PCLS required 48 h instead of 24 h of incubation with APAP and AMAP before substantial effects on the protein profiles in PCLS medium regarding toxicity could be observed.

Hepcidin, an iron-regulating hormone, was recently shown to be involved in APAP-mediated liver injury by increasing hepatic iron loading [23]. Plasma levels of hepcidin were reduced upon APAP treatment by the oxidative stress caused by APAP. In the current study, all drug treatments, except for AMAP in mouse PCLS, resulted in decreased hepcidin concentrations in PCLS medium samples of all species. For APAP and AMAP, this indicated that the same mechanisms as observed *in vivo* are likely to be involved in hepatotoxicty*in vitro*. For DF treatment, this suggests that oxidative stress might play a role in the mechanisms leading to liver injury [33, 34]. Although DF-treated human PCLS medium did not demonstrate any effects on SOD1 release, hepcidin concentration was decreased after 24 h of incubation. Hepcidin might, therefore, be involved early on in the manifestation of liver injury. Furthermore, LPS incubation induced hepcidin concentration dramatically, which is confirmative to what has been reported *in vivo*[22].

In conclusion, this is the first study on protein profiling in medium of human, mouse and rat PCLS, demonstrating that similar toxicity-related protein profiles were identified in mouse urine (*in vivo*) and in PCLS (*ex vivo*) prepared from mouse, rat and human. Hence, the PCLS system proves to be a promising, translational model for the identification of potential protein biomarkers, which could be developed as urinary biomarkers for human DILI, and provide insight into the mechanisms behind these adverse reactions.

Acknowledgements

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Mouse precision-cut liver slices as an *ex vivo* model to study idiosyncratic drug-induced liver injury

4

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Abstract

Idiosyncratic drug-induced liver injury (IDILI) has been the top reason for withdrawing drugs from the market or for black box warnings. IDILI may arise from the interaction of a drug's reactive metabolite with a mild inflammation that renders the liver more sensitive to injury resulting in increased toxicity (inflammatory stress hypothesis). Aiming to develop a robust *ex vivo* screening method to study inflammatory stress-related IDILI mechanisms and to find biomarkers that can detect or predict IDILI, mouse precision-cut liver slices (mPCLS) were co-incubated for 24h with IDILI-related drugs and lipopolysaccharide. Lipopolysaccharide exacerbated ketoconazole [15 μM] and clozapine [45 μM] toxicity but not their non-IDILI-related comparators, voriconazole [1500 μM] and olanzapine [45 μM]. However, the other IDILI-related drugs tested (diclofenac [200 μM], carbamazepine [400 μM] and troglitazone [30 μM]) did not cause synergistic toxicity with lipopolysaccharide after 24h incubation. Lipopolysaccharide further decreased the reduced glutathione levels caused by ketoconazole or clozapine in mPCLS after 24h incubation, which was not the case for the other drugs. Lipopolysaccharide significantly increased NO, cytokine and chemokine release into the mPCLS media, while the treatment with the drugs alone did not cause any substantial change. All seven drugs drastically reduced lipopolysaccharide-induced NO production. Interestingly, only ketoconazole and clozapine increased the lipopolysaccharide-induced G-CSF and GM-CSF release. Pilot experiments showed that diclofenac and troglitazone, but not carbamazepine, demonstrated synergistic toxicity with lipopolysaccharide after a longer incubation of 48h in mPCLS. In conclusion, we have developed an *ex vivo* model to detect inflammatory stress-related liver toxicity and identified ketoconazole, clozapine, troglitazone and diclofenac as drugs that showed synergistic toxicity with lipopolysaccharide. Reduced glutathione, G-CSF, and GM-CSF were identified to be potential biomarkers for IDILI-inducing drugs mediated by inflammatory stress and mPCLS appear to be a promising screening tool to further unravel the mechanism of IDILI.

1. Introduction

Idiosyncratic drug reactions (IDRs) can be defined as adverse drug reactions (ADRs) that occur in a small minority (0.01-1%) of the patients taking clinically-relevant doses and do not involve the known pharmacological effects of the drug.[1] IDRs are a major issue for drug development as over 10% of newly approved drugs in the US were withdrawn or restricted in use due to ADRs that were not predicted by pre-clinical and clinical trials.[2, 3] Drug-induced liver injury (DILI) has been the top reason for withdrawing drugs from the market.[4, 5] DILI accounts for more than 50% of acute liver failure cases and idiosyncratic DILI (IDILI) accounts for 13% of them.[3] It can be estimated that about 30,000 patients are required in clinical testing to detect IDILI that occurs in 1 in 10,000 patients.[3] Assuming a similar incidence in animals, 30,000 animals are needed for toxicity testing to detect IDILI. Because such large studies are not feasible, mechanisms of action of IDILI need to become better understood in order to develop IDILI predictive models and to identify biomarkers that can detect IDILI. The frequently stated notion that IDILI is dose-independent in fact refers to its response. However, the total dose has a bearing on IDILI as drugs that are administered at doses lower than 10 mg/day (e.g. OZ) are generally not associated with IDILI, while the typical IDILI-causing drugs (e.g. CZ) are given at higher doses.[6]

In many cases, metabolism of the drug to reactive metabolites and free radicals seems to be associated with DILI.[7] While it was suggested that reactive metabolites may also be responsible for many IDILI cases,[8, 9] there are also drugs that form reactive metabolites but are not related to any IDRs and IDR-related drugs that do not form reactive metabolites.[10] Several hypotheses regarding the mechanism of IDILI have been described extensively in recent reviews and many involve the formation of reactive metabolites.[1, 11, 12] Two of them, the danger hypothesis and the inflammatory stress hypothesis additionally propose inflammation as a cofounding factor for IDILI.[12] Such a reactive metabolite can bind to an endogenous protein forming a hapten,[13] which has been shown to trigger antibody formation. The danger hypothesis proposes that the formation of this hapten is insufficient to cause IDILI and a secondary "danger" signal is needed that acts as an adjuvant to initiate the injury.[14] This signal could be an inflammatory episode by bacterial or viral infection leading to cytokine release, which activates the immune system to induce allergic hepatotoxicity.[15] Alternatively, the inflammatory stress hypothesis suggests that an episode of mild inflammation could play a role in lowering the threshold for drug toxicity, thereby precipitating a toxic response and rendering an individual susceptible to IDILI.[16, 17]The low incidence of the concurrence of the danger signal with the drug exposure is supposed to be the basis of IDILI. Several animal models have been developed employing this hypothesis with the aim to mimic human IDILI, where drugs that cause human IDILI,

such as chlorpromazine, trovafloxacin and sulindac, were rendered more toxic in rats or mice by co-treatment with lipopolysaccharide (LPS), a potent inflammation inducer.[18-21]

The insights provided by animal models of IDILI could lead to the development of more robust *in vitro*/*ex vivo* predictive tests based on the elucidated mechanisms of action. One advantage of *in vitro*/*ex vivo* tests is the possibility to compare the toxicity and metabolism of drugs in animal and human. Precision-cut liver slices (PCLS) could be a promising *ex vivo* model to test this inflammatory stress hypothesis as PCLS retain the normal tissue architecture of a whole liver with all its cell types in their natural arrangement and integral intercellular interactions.[22-24] Phase I and II drug metabolism enzymes are active in PCLS and their metabolic functions and toxicity characteristics correlate with *in vivo* data.[24-29] Moreover, microarray analysis of xenobiotic-treated rat PCLS showed that they could mimic the toxicity as observed *in vivo* and discriminate between different mechanisms of hepatotoxicity.[30] It has also been shown that treatment of PCLS with LPS activated the Kupffer cells and induced an inflammatory response in the liver as evidenced by the production of nitric oxide (NO) and inflammatory cytokines, such as tumor necrosis factor-α (TNF) and interleukin 1β (IL-1β) both in rat and human liver slices,[31-33] indicating that the PCLS model can be used as translational model when animal and human tissue is employed.

In the present study, we investigated the toxicity of five drugs known to cause IDILI in patients: ketoconazole (KC), clozapine (CZ), diclofenac (DF), carbamazepine (CBZ), and troglitazone (TGZ) in the presence or absence of LPS in mPCLS. Additionally, non-IDILI-causing comparator drugs of KC and CZ in mice, voriconazole (VC) and olanzapine (OZ) respectively, were also investigated. VC and OZ are in the same chemical class of drugs as KC and CZ respectively, but do not induce liver toxicity in our model at the concentrations used. The viability of mPCLS was determined by means of adenosine triphosphate (ATP) content, leakage of lactate dehydrogenase (LDH), levels of reduced glutathione (GSH) and total glutathione, and histomorphology. We also examined each drug's effect on LPS-induced nitric oxide (NO), cytokine, and chemokine release in the mPCLS. The aim of our study was to test if mPCLS can be a suitable *ex vivo* model to study the inflammatory stress hypothesis, which of the IDILI-associated drugs could be identified with this model and to discover biomarker(s) that can identify drugs as potentially inducing IDR in mouse mPCLS.

2. Materials and Methods

2.1. Chemicals

Troglitazone (TGZ) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Ketoconazole (KC), clozapine (CZ), diclofenac sodium salt (DF), carbamazepine (CBZ) and

LPS derived from *Eschericia coli* serotype B55:O55 (Lot 050M4014, 600,000 EU/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Olanzapine (OZ) and voriconazole (VC) were kind gifts from Dr. Willem Schoonen (MSD, Oss, the Netherlands) and Jan-Willem Alffenaar (UMCG, Groningen, the Netherlands) respectively. All other drugs and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Stock solutions of each drug were made in DMSO (VWR, Briare, France) as the solvent.

2.2. Animals

Female C57BL/6 mice weighing 20-24 g were obtained from Harlan (Horst, the Netherlands). The mice were housed on a 12-h light/dark cycle in a temperature- and humidity-controlled room with food (Harlan chow no 2018, Horst, the Netherlands) and tap water *ad libitum*. The animals were allowed to acclimatize for at least seven days before experimentation. The experimental protocols were approved by the Animal Ethical Committee of the University of Groningen.

2.3. Excision of mouse liver

Under isofluorane/O_2 anesthesia, the liver was excised and placed into ice-cold University of Wisconsin (UW) organ preservation solution (DuPont Critical Care, Waukegab, IL, USA).

2.4. Preparation of the mPCLS

mPCLS were made as described previously for rat and human PCLS[34] with one minor modification: the cylindrical liver cores were made using a 5mm biopsy punch (Kai Industries, Seki, Japan) instead of using a drill. These cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs-Henseleit buffer saturated with carbogen (95% O_2 and 5% CO_2). PCLS (5 mm diameter, 200-300 μm thick and ca. 4.5-5.5 mg wet weight) were stored in ice-cold UW solution until incubation.

2.5. Incubation of the mPCLS

Incubation of mPCLS in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria) was performed as described before.[34] In brief, mPCLS were pre-incubated at 37°C for 1h individually in a well containing 1.3 ml Williams' medium E with glutamax-1 (Gibco, Paisley, UK), supplemented with 25 mM D-glucose and 50 μg/ml gentamicin (Gibco, Paisley, UK) (WEGG medium) in a 12-well plate with shaking (90 times/min) under saturated carbogen atmosphere. As was described for rat and human slices, pre-incubation allows

the mPCLS to restore their ATP levels (data not shown). After pre-incubation, mPCLS were transferred to fresh WEGG medium in the presence or absence of LPS (20,000 EU/ml) and in combination with KC [15 μM] or its analog VC [1500 μM], CZ [45 μM] or its analog OZ [45 μM], CBZ [400 μM], DF [200 μM], TGZ [30 μM] or the vehicle (final concentration of DMSO during incubation ≤0.5%) and incubated further for 24-48h without any further change in medium. The LPS and the drug were administered simultaneously. Pilot studies showed no differences in response when the drug was added 4h earlier or later than LPS. Preliminary dose-response studies were performed for LPS, with the objective to identify a low-toxic dose of LPS that generated significant inflammatory responses based on the measurements of pro-inflammatory cytokines. The concentrations of drugs used in this study were also selected from initial dose-response relationship studies based on the criteria that each drug concentration elicits slight drug-only hepatotoxicity. For all the IDILI-related drugs, the concentration was lower than 25-fold its plasma maximum concentration (C_{max}) value, whereas for the non-IDILI-related comparator drugs, voriconazole and olanzapine, a concentration of even ~100-fold C_{max} was selected. C_{max}values were obtained from a combination of literature searches and available databases, as in Xu et al.[35] Therapeutically appropriate drug exposure levels were defined by C_{max} values observed in humans upon single- or multi-dose administration. A concentration of 100-fold C_{max} was considered as a therapeutically relevant concentration limit for each drug incorporating a scaling factor to account for human population pharmacokinetic and toxicodynamicvariabilities[35]. Cosgrove *et al.* also used this parameter.[36]

2.6. ATP and protein content of mPCLS

Viability of mouse mPCLS after 24h incubation with various treatments was determined by measuring the ATP content of the mPCLS according to the method described earlier.[34] In brief, at the end of 24h incubation, three replicate mPCLS were collected individually in 1 ml 70% ethanol (v/v) containing 2 mM EDTA (pH 10.9) and snap-frozen in liquid nitrogen and stored at -80°C until analysis. The samples were homogenized using a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA) and centrifuged for 3 minutes at 13000 rpm and 4°C. The supernatant was diluted 10 times with 0.1 M TrisHCl containing 2 mM EDTA (pH 7.8) to reduce the ethanol concentration. The ATP content of the supernatant was measured using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) in a black 96-well plate Lucy1 luminometer (Anthos, Durham, NC, USA) using a standard ATP calibration curve.

The remaining pellet was used to determine the protein content of the mPCLS by dissolving the pellet in 200 μl of 5 M NaOH for 30 min. After dilution with water to a

concentration of 1 M NaOH, the protein content of the samples was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany) using bovine serum albumin (BSA) for the calibration curve.

Table 1. The levels of ATP, glutathione, NO, cytokines, and chemokines following 24h incubation of mPCLS in the absence (control) and presence of LPS. Data are given as mean ± SEM of at least 5 mice per group. Means of control and LPS-treated groups were compared by paired t-test, **p<0.01 and ***p<0.001 (LPS-treated mPCLS vs. control mPCLS).

	Control mPCLS	LPS-treated mPCLS
ATP (nmol/slice)	4.3 ± 0.2	3.6 ± 0.3**
Glutathione levels:		
- GSH (nmol/slice)	8.0 ± 0.8	10.1± 0.9
- Total glutathione (nmol/slice)	17.4± 0.6	19.9± 1.1
NO (μM)	3.5 ± 0.6	102 ± 11.6***
Cytokines:		
- IL-1β (pg/ml)	6.6 ± 2.1	65.6 ± 5.8***
- IL-6 (pg/ml)	101 ± 29.0	2220 ± 173***
- IFN-γ (pg/ml)	1.5 ± 0.4	132 ± 25.6***
- TNF-α (pg/ml)	22.5 ± 5.9	503 ± 47.6***
Chemokines		
- CCL3 (pg/ml)	94.6 ± 55.0	2490 ± 191***
- CCL5 (pg/ml)	41.6 ± 13.4	5420 ± 622***
- G-CSF (pg/ml)	26.6 ± 3.9	1200 ± 79.9***
- GM-CSF (pg/ml)	2.6 ± 0.9	59.2 ± 9.5***

2.7. LDH leakage of mPCLS

The leakage of the enzyme LDH to the media was assessed as an additional viability marker. For this assay, 50 μl of medium was collected from each of the three replicate mPCLS after 24h incubation and these three samples were mixed into one sample. The amount of LDH in the medium sample was analyzed using the Roche/Hitachi Modular system (Roche, Mannheim, Germany) according to a routine laboratory procedure. To determine the total LDH content in fresh mPCLS, three mPCLS were collected after 1h pre-incubation each

in 1.3 ml of fresh medium. They were homogenized using a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA) and centrifuged for 3 minutes at 13000 rpm and 4°C.The supernatant was then analyzed to determine the initial LDH content in fresh mPCLS.

2.8. Histomorphology of mPCLS

mPCLS, incubated with the drugs or solvent control for 24h, were subjected to morphological evaluation. mPCLS were fixed in 4% formaldehyde in phosphate-buffered saline solution for 24h at 4°C and stored in 70% ethanol at 4°C. Embedding in paraffin, sectioning (4 μm) and staining with hematoxylin and eosin (H&E) was performed as described previously.[37]

2.9. Glutathione content of mPCLS

Glutathione exists in the cells in reduced (GSH) and oxidized (GSSG) state. The GSH and total glutathione (GSH+GSSG) levels were measured in mPCLS following 24h incubation. Three replicate mPCLS were washed briefly in 0.9% NaCl solution, collected and snap-frozen in liquid nitrogen for further storage at -80°C until the analysis. Each slice was homogenized in 400 μL ice-cold 50 mMTris-HCl buffer, which contains 1mM EDTA (pH 7.4) and centrifuged for 3 minutes at 13000 rpm and 4°C. The supernatant was used for GSH and total glutathione assays separately using standard solutions of GSH.

To measure the content of total glutathione, 15 μL of 1mM NADPH solution (Roche Diagnostics GmbH, Manheim, Germany) and 7.5 μL of 20U/ml glutathione reductase solution (Sigma-Aldrich, St. Louis, MO, USA) were added to 150 μLof the supernatant and incubated for 15 min at 37ºC to reduce GSSG into GSH. 15 μL of 50% Trichloroacetic acid solution was used to precipitate the proteins which may disturb GSH measurement due to the presence of the SH-groups. After 5 min on ice, the samples were centrifuged for 5 min at 4°C at 4600 rpm for total GSH assay.

To measure reduced GSH, another 150 μL of the supernatant of the slice homogenate was taken to precipitate the proteins with 15 μL of 50% Trichloroacetic acid. After 5 min on ice, the samples were centrifuged for 5 min at 13000 rpm. From both samples, 50 μL of the supernatant was transferred to micro-titer 96-well plate and 200 μL of Ellman's reagent (1 mM solution of 5,5'-dithio-bisnitrobenzoic acid in 0.5 M Tris/10 mM EDTA buffer containing 10% v/v ethanol, pH 8.0) was added to each well and the absorbance was read at 405 nm after 5 minutes.

2.10. NO_x formation assay

The concentrations of nitrate and nitrite were determined in the media of mPCLS after 24h incubation. The assay involves enzymatic reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and

the NO production was measured as nitrate/nitrite (NO_x) concentrations in the media of mPCLS as described previously.[38]

2.11. Cytokine or chemokine measurement

The media after 24h mPCLS incubation were analyzed for cytokine production by cytometric bead array (CBA). The levels of ten pro-inflammatory cytokines/chemokines, namely, tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 12 (IL-12), CC chemokine ligand 3(CCL3), CC chemokine ligand 5(CCL5), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), were measured. These ten cytokines/chemokines were chosen because they are known to be involved in inflammatory reactions and the kits to measure them are commercially available. The measurement was done using mouse CBA flex sets (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction manual.

2.12. Statistics

Each experiment was performed with a minimum of 4 livers using three mPCLS for each experimental condition from each liver. Comparisons of two different groups were performed using two-tailed paired Student's t-test. P-value <0.05 was considered statistically significant. Comparisons between multiple groups were performed using two-way ANOVA using GraphPad Prism 5.0 statistics program (GraphPad Software Inc., San Diego, CA, USA). A level of significance of 5% was chosen to denote significant difference between means.

3. Results

3.1. Viability of mPCLS following 24h incubation

The viability of mPCLS was determined by comparing the ATP content of the treated mPCLS with that of control mPCLS and by comparing the percentage of LDH leaking to the media following 24h incubation (Figure 1). The average ATP content of mPCLS following 24h incubation was 4.3 nmol/slice (Table 1) or 7.0 nmol/mg protein. The average initial LDH activity in pre-incubated mPCLS is 2.75 mU/slice and approximately 11% of the LDH content in control mPCLS had leaked into the media after 24h incubation. Based on ATP content and/or LDH leakage of mPCLS, all IDILI-related drugs were used in doses that caused slight toxicity (max ~20% decrease in ATP) after 24h incubation without LPS. Nevertheless, the comparator drugs, VC and OZ were not toxic even at very high supra-

therapeutical concentrations (1500 and 45 μM respectively). LPS itself caused slight though significant toxicity in 24h-incubated mPCLS (15% loss of ATP content when compared with the control).

Synergistic toxicity was observed when the mPCLS were incubated with LPS+KC or LPS+CZ compared to LPS or KC or CZ alone. Their ATP levels were reduced below half of the value of the control mPCLS and more than 40% of the LDH content of the mPCLS leaked into the medium. This synergistic toxicity was substantial considering that treatments with KC or CZ or LPS alone only caused slight toxicity. In contrast, the respective comparator drugs VC and OZ did not show this phenomenon. However, LPS did not enhance the toxicity of the other 3 IDILI drugs DF, CBZ or TGZ in the mPCLS following 24h incubation.

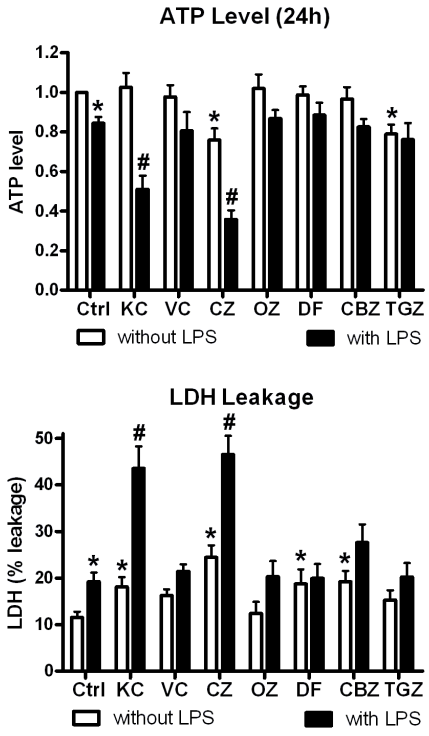

Figure 1. Viability of mPCLS indicated by the ATP content and LDH leakage after 24h incubation with various drugs in the absence (open bars) or presence (black bars) of LPS. ATP content is expressed as relative values to the control mPCLS without LPS-treatment, while the LDH leakage was expressed as the percentage of the total LDH content in a slice. Data represent the average ± SEM of 4-8 experiments (ATP) or 7-26 experiments (LDH), using 3 PCLS per experiment. *P<0.05 vs control by t-test and #P<0.05 by two-way ANOVA of the four different groups together, namely, the control, LPS-only, drug-only and LPS+drug groups.

The viability of mPCLS following 24h incubation was also assessed by histomorphology. The histomorphology data (Figure 2) confirmed the findings from the ATP and LDH data. mPCLS incubated with the combination of LPS and KC (Figure 2D) or CZ (Figure 2F) showed extensive necrotic areas as indicated by the massive loss of nuclei (dark purple dots), while incubation with LPS alone (Figure 2B), KC alone (Figure 2C) or CZ alone

(Figure 2E) showed only minor toxicity. Yet again, mPCLS that were treated with VC or OZ both in the absence and presence of LPS demonstrated similar morphology as the control mPCLS (Figure 2A) and LPS-treated mPCLS (Figure 2B) respectively (data not shown). There was also no significant difference between the histomorphology of mPCLS treated with TGZ (Figure 2G) or LPS+TGZ (Figure 2H). Similarly, 24h treatments with DF and CBZ did not show any significant difference in morphological damage in the absence or presence of LPS (data not shown).

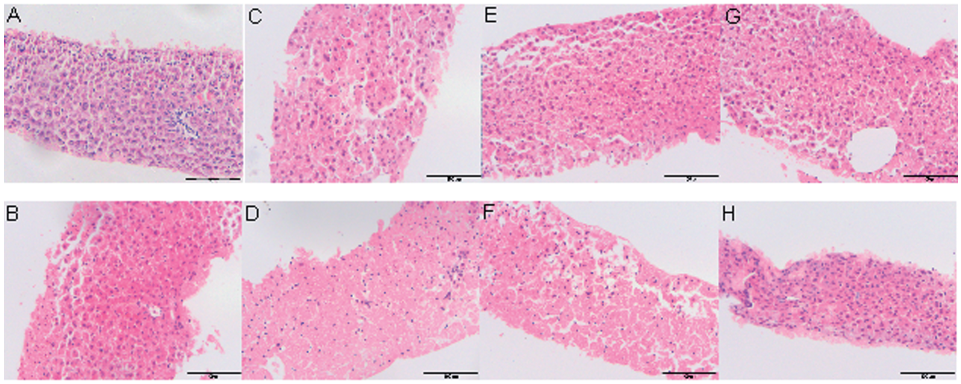

Figure 2. Histomorphology of mPCLS treated respectively with DMSO, KC, CZ, and TGZ in the absence of LPS (A, C, E, G) and in the presence of LPS (B, D, F, H) for 24h. Three individual experiments were performed with mPCLS. Representative images are shown. The sections were stained by hematoxylin-eosine, bar = 100 µm, magnification 200x.

3.2. Glutathione content of mPCLS

The reduced and total glutathione levels in mPCLS after 24h incubation were measured (Figure 3). When compared to the GSH content of the control mPCLS, all drugs caused a slight to significant reduction in the GSH level except for OZ that had no effect and TGZ, which increased the GSH level instead. Though not significant, LPS itself slightly increased both the levels of GSH and total glutathione. However, mPCLS co-incubated with LPS and either KC or CZ had significantly and substantially lower levels of both GSH and total glutathione when compared to the control, LPS-treated or KC- or CZ-treated mPCLS. The GSH levels in LPS+KT and LPS+CZ groups dropped to 14% and 21% of the control value respectively and the total glutathione concentrations of these groups were reduced into less than half of the concentration of the control. This reduction was not observed in the cases of the other drugs in combination with LPS and was only observed in the two drugs (KC and CZ) that caused synergistic toxicity with LPS (Figure 1).

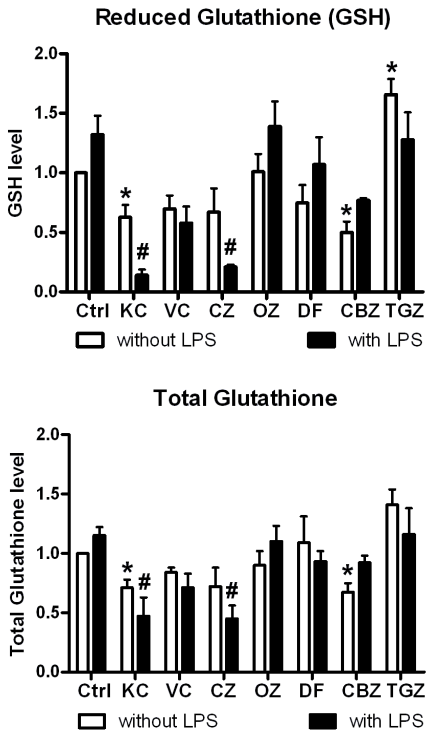

Figure 3. Reduced glutathione (GSH) and total glutathione levels in mPCLS following 24h incubation with various drugs in the absence (open bars) or presence (black bars) of LPS. The glutathione levels are expressed as relative values to the control, DMSO-treated mPCLS without LPS-treatment. Data represent the average ± SEM of 4-5 experiments using 3 mPCLS per experiment. *P<0.05 vs control by t-test and #P<0.05 by two-way ANOVA of four different groups together, namely, the control, LPS-only, drug-only and LPS+drug groups.

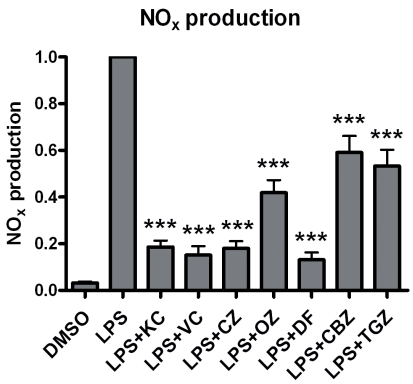

Figure 4. LPS-induced NO_x production by mPCLS co-incubated with various drugs for 24h. The level of NO_x production is expressed as relative values to the LPS-treated mPCLS. Treatmentwith any drug alone had no effect in NO_x concentration when compared to control mPCLS.Data represent the average ± SEM of 8-19 experiments, using 3 mPCLS per experiment. **P < 0.01 and ***P < 0.001 vs. LPS-treated group (t-test).

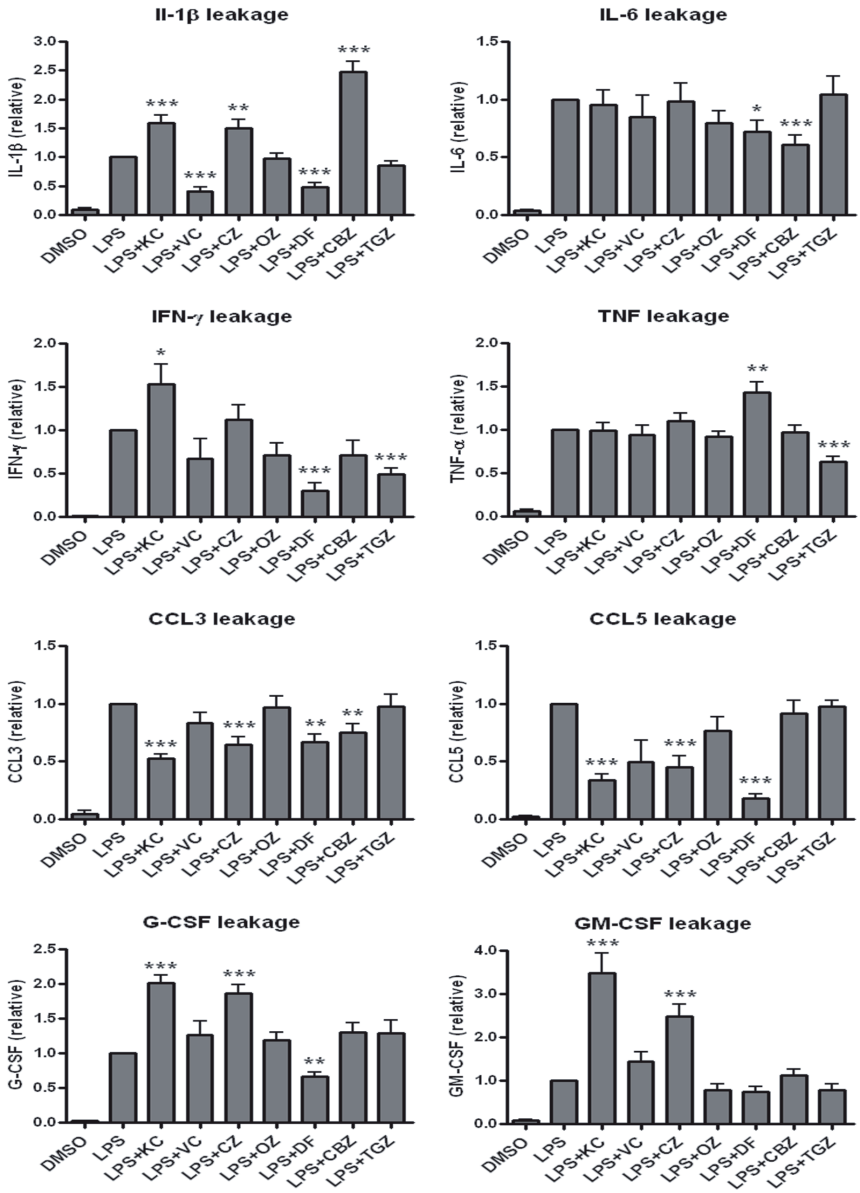

Figure 5. LPS-induced release of cytokines (IL-1β, IL-6, IFN-γ, TNF-α) and chemokines (CCL-3, CCL-5, G-CSF, GM-CSF) from mPCLS incubated with various drugs for 24h. The levels of cytokine or chemokine release are expressed as relative values to the LPS-treated mPCLS. Treatment with any drug alone had no effect in cytokine/chemokine release when compared to control mPCLS. Data represent the average ± SEM of 4-23 experiments, using 3 mPCLS per experiment. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. LPS-treated group (t-test).

3.3. LPS-induced NO production in mPCLS

LPS induced a significant NO production in mPCLS (Figure 4), measured as nitrate/nitrite (NO_x) concentrations in the media of the slices following 24h incubation. Treatments of PCLS with any drug alone did not result in a significant change in NO_x concentration when compared to control mPCLS. However, all IDILI-drugs and comparator drugs tested reduced the LPS-induced NO production extensively. The levels of LPS-induced NO production were reduced in the range from 41% by CBZ to 87% by DF of the control value (treatment with LPS alone).

3.4. LPS-induced release of cytokines and chemokines in mPCLS

LPS induced significant release of cytokines or chemokines in mPCLS (Figure 5). The release of ten pro-inflammatory cytokines (IL-1β, IL-6, IL-10, IL-12, IFN-γ, TNF-α) and chemokines (CCL-3, CCL-5, G-CSF, GM-CSF) were measured in the media following 24h incubation of the mPCLS. IL-1β, IL-6, IFN-γ, TNF-α CCL-3, CCL-5, G-CSF, GM-CSF were excreted into the medium after treatment with LPS. IL-10 and IL-12 leakage could not be quantified because the concentrations were below the detection level and thus no data could be shown for these cytokines. Treatment of mPCLS with any of the drugs alone did not result in a significant change in cytokine or chemokine release when compared to control mPCLS. In general, all drugs influenced the LPS-induced release of some of the cytokines or chemokines in mPCLS. KC, CZ, and CBZ significantly increased the LPS-induced IL-1β release, while DF and VC significantly decreased it. Both DF and CBZ reduced the LPS-induced IL-6 release. LPS-induced IFN-γ release was enhanced only by KC, while OZ, DF, and TGZ attenuated it. DF was the only drug that increased the release of TNF-α, while TGZ was the only drug that decreased it. Moreover, KC, CZ, and DF substantially reduced the LPS-induced CCL3 and CCL5 release, while CBZ reduced only the CCL3 release. Interestingly, KC and CZ, the only two drugs that showed synergistic toxicity with LPS, were also the only drugs that significantly increased the LPS-induced release of G-CSF (~2.5 and ~3.5 folds respectively) and GM-CSF (~2 fold). In contrast, the other drugs did not have any influence on their release, except for DF, which even decreased the G-CSF release.

3.5. Viability of mPCLS following 48h incubation

The lack of synergistic toxicity in mPCLS co-incubated with LPS and either DF or CBZ or TGZ after 24h was unexpected and therefore, we incubated the mPCLS for a longer period i.e. 48h (Figure 6). The average ATP content of mPCLS following 48h incubation was 4.3 nmol/slice, which was the same content as after 24h incubation. Two of the IDILI-related

drugs that did not cause synergistic toxicity with LPS after 24h incubation, namely DF and TGZ, demonstrated synergistic toxicity with LPS following 48h incubation based on the ATP contents of mPCLS. The contents of the ATP in mPCLS co-incubated with LPS+DF and LPS+TGZ were reduced to below 45% of the total content of control PCLS. However, even after 48h, LPS still did not enhance the toxicity of carbamazepine.

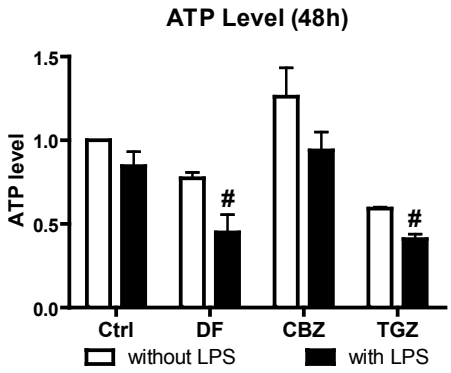

Figure 6. Viability of mPCLS indicated by the ATP content after 48h incubation with DF (200 μM), CBZ (400 μM), and TGZ (30 μM) in the absence (open bars) or presence (black bars) of LPS (30 μg/ml). ATP content is expressed as relative values to the control mPCLS without LPS-treatment. Data represent the average ± SEM of 4 experiments, using 3 PCLS per experiment. *P<0.05 vs control by t-test and #P<0.05 by two-way ANOVA of the four different groups together, namely, the control, LPS-only, drug-only and LPS+drug groups.

4. Discussion

IDILI has been difficult to predict and prevent due to its relatively rare occurrence, its relatively unknown mechanisms, and the lack of reliable screening methods.[39] Therefore, it is important to develop robust and accurate screening methods for early detection of IDILI in pre-clinical development since every new drug has a potential IDILI liability.[40] Therefore, we investigated the suitability of mPCLS for this purpose. Female mice might be a better model than male mice as DILI in human is more common among women.[41] Roth and Ganey proposed that a good model for IDILI should have liver injury as the major endpoint, since it is hepatotoxicity in humans that is being modeled.[42] They used LPS and drug co-administration in rat and mouse *in vivo* as a model for the inflammatory stress hypothesis and found indeed synergistic toxicity with some of the well-known IDILI drugs. The use of PCLS as an *ex vivo* model to test this hypothesis has a disadvantage i.e. the absence of hepatic neutrophil accumulation and infiltration after LPS treatment. Neutrophils were found to be a mediator of injury in LPS-potentiated toxicity model.[19, 43,

[44] However, it cannot be excluded that the neutrophils present in the liver tissue during harvesting may play a role in the synergistic toxicity in PCLS. An important impediment to developing animal models of IDILI is the lack of possibilities to unravel the mechanisms of these reactions in humans.[42] In order to develop an ex vivo model for IDILI that represents the liver histology and all the cells present in the liver, we studied co-administration of LPS and drugs in the PCLS. This is the first attempt to study idiosyncratic drug reactions in PCLS. An additional advantage of an *ex vivo* model is that it contributes to the reduction in animal use and has the potential to compare animal and human tissue.[34]

Five reactive metabolite-producing hepatotoxicants, which are known to cause IDILI in humans (along with two non-toxic comparator drugs), were tested in the absence or presence of LPS in mPCLS system. The concentrations of the IDILI-drugs used in this study were chosen to induce a low level of toxicity and are below 25*C_{max} in human plasma, which is considered physiologically relevant[35] and to obtain a low false positive rate as many non-hepatotoxic drugs induce hepatocyte death at concentrations exceeding 100*C_{max}.[36]The concentration of KC (15 μM) used here was similar to its C_{max}, while the CZ concentration used (45 μM) was approximately 15*C_{max}. Interestingly, there was no toxicity or synergistic toxicity attributed to VC or OZ, the comparator drugs of KC and CZ, even though much higher relative concentrations (approximately ~100*C_{max}) of VC (1500 μM) or OZ (45 μM) were used. Based on the ATP levels and LDH leakage, mPCLS co-incubated with LPS and KC or CZ showed synergistic hepatotoxicity when compared to the mPCLStreated with LPS or drug alone (Figure 1). The histomorphology data confirmed this finding as extensive necrotic areas can be seen in mPCLS co-incubated with LPS and KC or CZ (Figure 2).Our results are in line with the results obtained *in vivo* where co-exposure of LPS and trovafloxacin, but not levofloxacin, caused IDILI both in mice[20] and rats.[19] Additionally, co-exposure of LPS and monocrotaline in rats also resulted in synergistic hepatotoxicity and inhibition of Kupffer cell function attenuated the injury suggesting that Kupffer cells are important mediators in this synergistic toxicity.[43] This is in line with the observation that LPS significantly enhanced ranitidine toxicity, but not its corresponding negative control famotidine, in 3D co-cultures of hepatocytes and non-parenchymal cells to a much higher extent than in a monoculture containing mainly hepatocytes.[45] In addition, Cosgrove *et al.* also observed dose-dependent hepatotoxicity synergies in primary rat hepatocytes between LPS in the presence of a pro-inflammatory cytokines mix and multiple IDILI-related drugs (e.g. ranitidine and trovafloxacin) and not with the non-toxic comparator drugs (e.g. cimetidine and levofloxacin).[36] These synergistic hepatotoxicity phenomena have been described from the standpoint of an inflammatory stress caused by LPS enhancing the toxicity of a drug. However, it is equally plausible

that a drug could enhance the sensitivity of the liver to a subtoxic dose of a potentially hepatotoxic inflammagen such as LPS.[46]

Unexpectedly, in our study co-exposure of LPS and three IDILI-related drugs (DF, CBZ, and TGZ) did not show synergistic hepatotoxicity in mPCLS after 24h incubation (Figure 1). Subsequently, we did a pilot experiment to test whether prolonged incubation would reveal this synergism. ATP content of mPCLS following 48h incubation with TGZ and DF, but not CBZ, in the presence of LPS indeed showed synergistic toxicity (more than 50% depletion of ATP) (Figure 6). These data show that different drugs may require a different exposure time to develop synergistic toxicity, which is in line with the data of Roth and Ganey, who showed that various doses of LPS and various administration times of LPS and drug might be needed to observe this synergistic hepatotoxicity *in vivo* for different drugs.[42] In addition lack of synergistic effects of LPS with CBZ may be due to species differences in sensitivity for IDILI effects of these drugs. The drugs selected for this study are well known IDILI drugs in human, but it remains unknown whether these drugs would also induce IDILI in mice as to the best of our knowledge no data were published on the effects of CBZ and LPS in mouse *in vivo*. Idiosyncratic reactions can even be strain dependent as a combination of penicillamine and poly-IC induced autoimmunity in Brown Norway rats but not in Lewis rats.[47] An alternative explanation for this lack of synergistic toxicity of LPS with CBZ might be that this drug causes IDILI via another mechanism than the one proposed in the inflammatory stress hypothesis. A model based on a single hypothesis might not predict adequately all drugs causing IDILI in humans as it is likely that different IDILI-related drugs would exert their toxicity via different mechanisms of action and even various mechanisms may be involved with IDILI from a single drug.[42]

The depletion of GSH has been suggested as cofounding factor in addition to the formation of reactive metabolites in the development of IDILI.[48] Based on an observation that LPS treatment *in vivo* decreased the intracellular GSH concentration in rat livers,[49] we investigated the amount of GSH and total glutathione in mPCLS following 24h incubation. In contrast to their finding, LPS did not reduce the GSH concentration but there was even a tendency that LPS slightly increased the GSH concentration in mPCLS (Figure 3). Interestingly, this increase was also found in rat Kupffer cells and endothelial cells in vivo after LPS treatment.[50] The synergistic toxicity caused by LPS and KC or CZ may be explained by the substantially lower GSH levels in mPCLS co-incubated with KC or CZ in the presence of LPS when compared to the ones incubated without LPS (Figure 3). It might be of interest to investigate in the future whether the metabolism of these drugs is changed and particularly if GSH conjugation is increased when these drugs are dosed together with LPS. However, similar experiments performed in our laboratory with human PCLS

indicated that co-exposure of the slices to LPS and CZ also resulted in lower GSH levels but did not change the types and the amounts of CZ phase 1 metabolites nor did it change the amount of GSH or cysteine conjugates formed compared to CZ alone (manuscript in preparation).

Mechanisms by which interactions between LPS and xenobiotics occur are largely unknown.[51] In light of this, we investigated the drugs' effects on LPS-induced inflammatory responses. LPS has been shown to induce substantial NO production in rat PCLS.[31] Accordingly, the NO_x concentration was almost 30-fold upregulated in the LPS-treated mPCLS (Table 1). It was reported that NO might play a protective role in LPS-induced liver injury because nonselective inhibitors of NO synthase exacerbated the toxicity.[52]However, NO produced by the inducible NO synthase can either be detrimental or protective to the liver depending on the conditions.[53] Interestingly, all seven drugs tested attenuated LPS-induced NO production (Figure 4). Moreover, we did not observe any difference in the extent of reduction of NO production between the drugs that showed synergistic hepatotoxicity with LPS (KC, CZ), and their non-IDILI-causing comparators (VC, OZ), and with the other IDILI drugs (DF, CBZ, TGZ) (Fig 4). Based on these results, we concluded that NO does not play a role in inflammatory stress-related mechanism of IDILI in mPCLS.

Cytokines or chemokines that are derived primarily from Kupffer cells might be critical inflammatory mediators that contribute to the synergistic toxic responses in the LPS+drug model.[51] LPS has been shown to elevate the release of inflammatory cytokines in both rat[31] and human[33] PCLS. Correspondingly, LPS substantially induced the cytokine or chemokine release in mPCLS (Table 1). TNF-α is probably the most widely studied cytokine that plays a critical role in inflammatory stress-related IDILI mechanism.[44] Ranitidine treatment *in vivo* caused the LPS-induced serum TNF-α concentration increase to last longer than in rats given LPS alone and LPS/ranitidine-co-treated rats developed synergistic hepatotoxicity,[54] although it is still controversial whether ranitidine causes IDILI in man.[11] Additionally, trovafloxacin administered prior to LPS caused significant hepatotoxicity and greater elevation of TNF-α and IFN-γ concentrations in the plasma compared to LPS-treated mice, while levofloxacin co-treatment had no toxicity and no effect on LPS-induced changes in plasma TNF-α or IFN-γ concentration.[20, 55] Moreover, the IFN-γ-/- mice, which have decreased plasma concentrations of TNF-α and IL-1β when compared to wild-type mice after LPS+trovafloxacin treatment, were significantly protected from LPS+trovafloxacin-induced liver injury.[55] It was also shown that administration of TNF-α instead of LPS to rats in vivo could induce inflammatory reactions that potentiated the hepatotoxicity of trovafloxacin in rats, although the pattern of mRNA gene expression changes was only partly overlapping suggesting that the TNF-α mediated inflammatory

reaction is not identical to the LPS-mediated reaction.[56] Our results indicate that none of the drugs change the LPS-induced TNF-α release in mPCLS except TGZ that decreased it and DF that elevated it (Figure 5).This may indicate that although TNF-α is important for the inflammatory reaction, synergistic toxicity in mPCLS is not related to the level of TNF-α per se. DF decreased the LPS-induced release of all other cytokines and chemokines. This is in line with the anti-inflammatory effects exerted by DF as a non-stereoidal anti-inflammatory drug (NSAID). DF inhibits prostaglandin synthesis by the inhibition of cyclooxygenase (COX).[57] COX-2 is expressed in the liver almost exclusively in the non-hepatocyte cell population and under rapid response to a pro-inflammatory challenge.[58] The non-IDILI-causing OZ and VC only reduced the LPS-induced release of IFN-γ and IL-1β respectively. DF and TGZ reduced both the LPS-induced IFN-γ and IL-6 release in mPCLS, while KC was the only drug to increase the IFN-γ release. Taken the IFN- γ data together, we cannot conclude that increased IFN- γ plays a major role in the development of the synergistic toxicity in mPCLS. Moreover, KC and CZ, the two drugs that showed synergistic hepatotoxicity with LPS, increased LPS-induced IL-1β release, which may suggest a role for IL-1 β in the synergistic effect. However, CBZ, which did not show synergistic toxicity, also increased it, even to a higher extent. Furthermore, a pilot study showed that anti-IL-1β neutralizing antibody did not reduce the synergistic toxicity after LPS+KC or LPS+CZ co-exposure (unpublished data). The chemokine measurement data revealed that IDILI-related drugs except TGZ actually decreased LPS-induced release of CCL3 and/or CCL5, while the non-IDILI-related drugs did not change the LPS-induced release of these chemokines. Further studies are needed to investigate if these chemokines may be potential biomarkers for IDILI. Interestingly, we found a correlation between the synergistic hepatotoxicity and the elevation of LPS-induced release of G-CSF and GM-CSF in mPCLS (Figures 1 & 5) as KC and CZ were the only two drugs that increased the LPS-induced release of these chemokines. This is in line with the findings of Waring et al. that the mRNA expression of GM-CSF was also significantly upregulated in rats co-treated with LPS and trovafloxacin but not with LPS+levofloxacin or LPS alone when compared to the control rats[19]Moreover, the toxicity of LPS was increased in mice pre-treated with GM-CSF.[59] But up to now, the role of G-CSF in IDILI has not been investigated. These data altogether may indicate a potential role for G-CSF and GM-CSF as biomarkers to identify drugs that cause IDILI via an inflammatory stress related mechanism.

In conclusion, we have developed an *ex vivo* model utilizing mPCLS to detect inflammatory stress-related IDILI. Four of the five tested IDILI-associated drugs (KC and CZ after 24 h and DF and TGZ after 48h) were identified by this model by showing synergistic toxicity with LPS according to the inflammatory stress hypothesis. For KC and

CZ, the synergistic toxicity phenomenon after 24h was accompanied by synergistic GSH depletion and upregulation of LPS-induced G-CSF and GM-CSF release in mPCLS after co-incubation with LPS. Therefore, GSH, G-CSF, and GM-CSF are potential biomarkers in this model to detect IDILI related to inflammatory stress. Further research should involve the measurement of these markers in mPCLS after 48h incubation and also the inclusion of more IDILI-related drugs together with their non-IDILI-related comparator drugs to be tested in mPCLS. An important advantage of this PCLS model is the possibility of using human tissue to detect species differences and to better mimic IDILI reactions in humans. Therefore, the PCLS system may be a prospective *ex vivo* translational screening tool that can be utilized in the preclinical and clinical settings to characterize IDILI at various stages of drug development.

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Human precision-cut liver slices as an *ex vivo* model to study idiosyncratic drug-induced liver injury

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Abstract

Idiosyncratic drug-induced liver injury (IDILI) is a major problem during drug development and has caused drug withdrawal and black-box warnings. Due to the low concordance of the hepatotoxicity of drugs in animals and humans, robust screening methods using human tissue are needed to predict IDILI in human. According to the inflammatory stress hypothesis, the effects of inflammation interact with the effects of a drug or its reactive metabolite precipitating toxic reactions into the liver. As a follow–up of our recently published mouse precision-cut liver slices (PCLS) model, an *ex vivo* model involving human precision-cut liver slices (hPCLS), co-incubated for 24h with IDILI-related drugs and lipopolysaccharide (LPS), was developed to study IDILI mechanisms related to inflammatory stress in humans and to detect potential biomarkers. LPS exacerbated ketoconazole and clozapine toxicity but not their non-IDILI-related comparators, voriconazole and olanzapine. However, the IDILI-related drugs diclofenac, carbamazepine and troglitazone did not show synergistic toxicity with LPS after 24h incubation. Coincubation of ketoconazole and clozapine with LPS decreased the glutathione levels in hPCLS, but not for the other drugs. All drugs affected LPS-induced cytokine release, but interestingly, only ketoconazole and clozapine increased the LPS-induced TNF release. Decreased glutathione- and cysteine-conjugates of clozapine were detected in IDILI-responding PCLS following cotreatment with LPS. In conclusion, we identified ketoconazole and clozapine as drugs that exhibited synergistic toxicity with LPS, while glutathione and TNF were found to be potential biomarkers for IDILI-inducing drugs mediated by inflammatory stress. hPCLS appear suitable to further unravel the mechanisms of inflammatory stress-associated IDILI.

1. Introduction

Drug-induced liver injury (DILI) is the number one cause of acute liver failure that often results in liver transplantation or death[1]and it is also the top reason for drugs' black box warnings and withdrawal from the market [2]. The low predictivity of DILI relates back to the preclinical and clinical trials of such xenobiotics, as in general, the concordance rate of toxicity of drugs in animals and humans is distressingly low, being only 43% for rodent species alone. Hepatotoxicity is one of the two human toxicities with the poorest correlation with animal studies despite its relatively high incidence [3]. Therefore, the prediction of DILI relies largely on clinical studies and human *in vitro* studies. Even more difficult is the prediction of idiosyncratic DILI (IDILI). IDILI occurs only in a small subset of the population (0.01-1%) and its mechanisms of action have not been elucidated, although it is generally accepted that it is not related to the pharmacology of the drugs [4]. It was estimated that to detect IDILI that occurs in 1 in 10,000 patients, approximately 30,000 patients are required in clinical testing [5]. The low incidence of this IDILI reaction coupled to the insufficient predictive power of the current methods makes it virtually impossible to detect IDILI during preclinical and clinical trials.

There are several proposed mechanisms of action of IDILI, which have been described extensively in recent reviews and many involve the formation of reactive metabolites [4, 6-9], but not all drugs that form reactive metabolites are associated with IDILI [10]. Another common finding during IDILI is the presence of hepatic inflammation [11], which might originate from bacterial or viral infection leading to cytokine release [12]. This inflammatory episode could play a role in lowering the threshold for drug toxicity, thereby precipitating a toxic response and rendering an individual susceptible to IDILI [13, 14]. This has been the basis of both the inflammatory stress hypothesis [13, 15] and the danger hypothesis [16, 17]. Several animal models have been developed based on this hypothesis with the aim to mimic IDILI, where IDILI-related drugs, such as chlorpromazine, trovafloxacin and sulindac, were rendered more toxic in rats or mice *in vivo* during inflammation, for instance by co-treatment with lipopolysaccharide (LPS), a potent inflammation inducer [18-21]. The insights provided by these animal models of IDILI could lead to the development of human IDILI-predictive models to be used during preclinical studies to detect and eliminate new drug candidates which have the potential to cause human IDILI. Recently, we have developed an *ex vivo* model to test the inflammatory stress hypothesis for inflammation-induced IDILI and found synergistic toxicity when mouse precision-cut liver slices (PCLS) were coincubated with LPS and 4 out of 5 IDILI-associated drugs, but not with the pharmacologically-similar comparator drugs that have not been associated with IDILI [22].

These PCLS represent an *in vitro* system which retains the normal tissue architecture of an intact liver with all its cell types in their natural arrangement and integral intercellular and cell-matrix interactions [23-25]. They are metabolically fully competent with active phase I and II drug metabolism enzymes during 24 h of incubation [25-30]. The gene expression profile of PCLS was shown to have a higher similarity to intact liver compared to primary hepatocytes and cell lines [31]. Moreover, microarray analysis of xenobiotic-treated rat PCLS showed that they could mimic the toxicity as observed *in vivo* and discriminate between different mechanisms of hepatotoxicity [32]. Relatively stable expression of genes involved in drug transport, metabolism and toxicity during normal incubation of human PCLS for 24 h was reported, whereas incubation with acetaminophen caused significant changes in gene expression [33]. It has also been shown that the Kupffer cells in PCLS can be activated by LPS treatment inducing an inflammatory response as evidenced by the production of nitric oxide (NO) and inflammatory cytokines, such as tumor necrosis factor-α (TNF) and interleukin 1β (IL-1β) and IL-6 [22, 34-36]. The objectives of our study were to test if hPCLS can be a suitable *ex vivo* model to study the inflammatory stress hypothesis in humans, which of the IDILI-associated drugs could be identified with this model and to discover biomarker(s) that could be used to predict human IDILI in a translational model.

Although there is no evident dose-effect relationship for drugs causing IDILI, which occurs often at therapeutical doses, drugs administered at doses lower than 10 mg/day are generally not associated with IDILI [37]. In this study, we investigated whether cotreatment with LPS could aggravate the toxicity of five reactive metabolite-producing human IDILI drugs that are used at doses higher than 10 mg/day, namely, ketoconazole (KC), clozapine (CZ), diclofenac (DF), carbamazepine (CBZ), and troglitazone (TGZ) in hPCLS. Additionally, voriconazole (VC) and olanzapine (OZ) were investigated as comparator drugs of KC and CZ, respectively, which are in the same chemical class of drugs as KC and CZ respectively, but are not clinically associated with IDILI. VC causes dose-dependent hepatotoxicity that occurs in approximately 51% of patients [38, 39], but is not known to be idiosyncratic. OZ is not associated with DILI or with any idiosyncratic reaction and is commonly used as the negative control for CZ [40]. The effect of cotreatment with LPS on the viability of hPCLS was determined by means of adenosine triphosphate (ATP) content, levels of reduced glutathione (GSH) and total glutathione, and histomorphology. As the inflammatory reaction was hypothesized to play a role in IDILI, we also examined each drug's effect on LPS-induced release of soluble intercellular adhesion molecule-1 (sICAM-1) and cytokines in the media of hPCLS. The results were compared with the previously obtained data from mouse PCLS [22].

2. Materials and Methods

2.1. Chemicals

Troglitazone (TGZ) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Ketoconazole (KC), clozapine (CZ), diclofenac sodium salt (DF), carbamazepine (CBZ) and LPS derived from *Eschericia coli* serotype B55:O55 (Lot 050M4014, 600,000 EU/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Olanzapine (OZ) and voriconazole (VC) were kind gifts from Dr. Willem Schoonen (MSD, Oss, the Netherlands) and Jan-Willem Alffenaar (UMCG, Groningen, the Netherlands) respectively. All other drugs and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Stock solutions of each drug were made in DMSO (VWR, Briare, France) as the solvent.

2.2. Human liver tissue

Pieces of human liver tissue were obtained from patients undergoing partial hepatectomy (PH) for the removal of carcinoma or from liver tissue remaining as surgical waste after split liver transplantation (TX), as described previously [33]. The experimental protocols were approved by the Medical Ethical Committee of the University Medical Center Groningen. The liver tissue used in this study originated from 32 donors, of which 12 were male and 20 were female, with age range from 17 to 76 (Table 1). The number of drugs that could be tested simultaneously in one liver varied and was limited by the size of the tissue sample and thus by the number of PCLS that could be prepared.

Table 1. Summary of human liver donor characteristics used in this study (n≥5 for each endpoint), PH is liver tissue after partial hepatectomy, TX is liver tissue remaining from donor liver after transplantation. The sex and the age range of the donors (with the average inside brackets) are identified. The ATP content of human PCLS after 24h of incubation (with the average inside brackets) is also indicated. The responder livers do not correlate with sex or age or type of liver.

Total Liver Used	PH / TX	Male / Female	Age range (years old)	ATP at 24h range (nmol/mg protein)
32	17 PH and 15 TX	20 M and 12 F	17 – 76 (50)	2.0 - 11.4 (7.8)

2.3. Preparation of the hPCLS

hPCLS were made as described previously for human PCLS [41]. Pieces of human liver obtained after partial hepatectomy were perfused with cold University of Wisconsin (UW) organ preservation solution (DuPont Critical Care, Waukegab, IL, USA) immediately after removal from the body. Cores with a diameter of 5mm were punched out of the tissue and these cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs-Henseleit buffer saturated with carbogen (95% O_2 and 5% CO_2). PCLS (5 mm diameter, 200-300 μm thick and ca. 4.5-5.5 mg wet weight) were stored in ice-cold UW solution until incubation.

2.4. Incubation of the hPCLS

Incubation of hPCLS in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria) was performed as described before [41]. In brief, hPCLS were pre-incubated at 37°C for 1h individually in a well containing 1.3 ml Williams' medium E with glutamax-1 (Gibco, Paisley, UK), supplemented with 25 mM D-glucose and 50 μg/ml gentamicin (Gibco, Paisley, UK) (WEGG medium) in a 12-well plate with shaking (90 times/min) under saturated carbogen atmosphere. Pre-incubation for 1 h allows the hPCLS to restore their ATP levels (data not shown) and removes cell debris from the cutting surfaces. After pre-incubation, the hPCLS were transferred to fresh WEGG medium in the presence or absence of LPS (24,000 EU/ml) and in combination with KC [10 μM] or its analog VC [250 μM], CZ [60 μM] or its analog OZ [60 μM], CBZ [400 μM], DF [400 μM], TGZ [15 μM] or the vehicle (final concentration of DMSO during incubation ≤0.5%) and incubated further for 24h without any further change in medium. The LPS and the drug were administered simultaneously. Pilot studies showed no differences in response when the drug was added 4h earlier or later than LPS. Preliminary dose-response studies were performed for LPS, with the objective to identify a dose of LPS that generated significant inflammatory responses based on the measurements of pro-inflammatory cytokines that caused minimal toxicity. The concentrations of drugs used in this study were also selected from initial concentration-response relationship studies, where we generally selected a concentration that elicits no or minimal drug-only hepatotoxicity. The concentrations used were all physiologically relevant as for each drug it was lower than 20-fold its plasma maximum concentration (C_{max}) value, observed in humans upon single- or multi-dose administration with the exception of the concentrations chosen for diclofenac (~50-fold C_{max}) and olanzapine (~150-fold C_{max}). Similar relative concentrations were previously employed in *in vitro* / *ex vivo* studies [22, 42, 43]. C_{max} values were obtained from a combination of literature searches and available databases, as in Xu et al [42].

2.5. Viability of hPCLS

Viability of the hPCLS after 24h incubation with various treatments was determined by measuring the ATP content according to the method described earlier [41]. In brief, at the end of 24h incubation, three replicate hPCLS were collected individually in 1 ml 70% ethanol (v/v) containing 2 mM EDTA (pH 10.9) and snap-frozen in liquid nitrogen and stored at -80°C until analysis. The samples were homogenized using a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA) and centrifuged for 3 minutes at 13000 rpm and 4°C. The supernatant was diluted 10 times with 0.1 M Tris HCl containing 2 mM EDTA (pH 7.8) to reduce the ethanol concentration. The ATP content of the supernatant was measured using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) in a black 96-well plate Lucy1 luminometer (Anthos, Durham, NC, USA) using a standard ATP calibration curve.

The remaining pellet was used to determine the protein content of the hPCLS by dissolving the pellet in 200 µl of 5 M NaOH for 30 min. After dilution with water to a concentration of 1 M NaOH, the protein content of the samples was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany) using bovine serum albumin (BSA) for the calibration curve.

Leakage of enzymes (LDH, ALT, and AST) to the media of hPCLS, was measured but the data obtained was inconclusive as the results were very variable and differences between control and treated hPCLS were not significant. This is in contrast to our mouse studies where the leakage of LDH was a sensitive method to measure viability in mouse PCLS [22]. This may be caused by the larger individual variability in the human liver samples.

2.6. Histomorphology of hPCLS

hPCLS, incubated with the drugs or solvent control for 24h, were subjected to morphological evaluation. hPCLS were fixed in 4% formaldehyde in phosphate-buffered saline solution for 24h at 4°C and stored in 70% ethanol at 4°C. Embedding in paraffin, sectioning (4 µm) and staining with hematoxylin and eosin (H&E) was performed as described previously [44].

2.7. Glutathione content of hPCLS

Both reduced glutathione (GSH) and total (reduced plus oxidized (GSSG)) glutathione levels were measured in hPCLS following 24h incubation as described previously [22].

2.8. sICAM-1 and cytokine measurement

The media after 24h hPCLS incubation were analyzed for the release of sICAM-1 and cytokines by cytometric bead array (CBA). The levels of nine pro-inflammatory cytokines, namely, interferon gamma (IFN-γ), interleukin-1beta (IL-1β), interleukin-10 (IL-10), interleukin-12 (IL-12), tumor necrosis factor alpha (TNF), CC chemokine ligand 3(CCL3), CC chemokine ligand

5(CCL5), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), were measured. sICAM-1 and these nine cytokines were chosen because they are known to be involved in inflammatory reactions and the kits to measure them are commercially available. The measurement was done using mouse CBA flex sets (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction manual.

2.9. Metabolite measurement of CZ-treated hPCLS

The metabolites of CZ were analyzed in the media following 24h incubation of hPCLS in the absence or presence of LPS by reversed-phase liquid chromatography as described previously [45].

2.10. Statistics

Each experiment was performed with a minimum of 5 livers using three hPCLS for each experimental condition from each liver. Comparisons of two different groups were performed using two-tailed paired Student's t-test. P-value <0.05 was considered statistically significant. Comparisons between multiple groups were performed using two-way analysis of variance (ANOVA) using GraphPad Prism 5.0 statistics program (GraphPad Software Inc., San Diego, CA, USA). A level of significance of 5% was chosen to denote significant difference between means.

3. Results

3.1. Viability of human PCLS

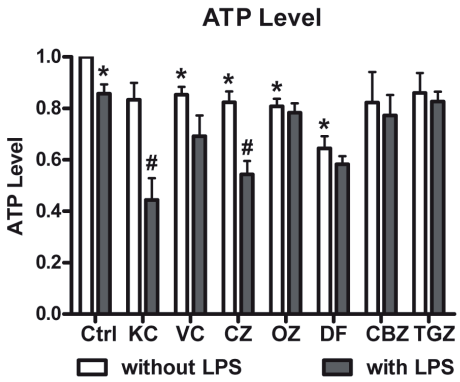

Figure 1. Viability of hPCLS indicated by the ATP content after 24h incubation with various drugs in the absence (open bars) or presence (filled bars) of LPS. ATP content is expressed as relative values to the control hPCLS without LPS-treatment. Data represent the average ± SEM of 5-14 experiments, using 3 PCLS per experiment. *P<0.05 vs control by t-test and #P<0.05 by two-way ANOVA of the four different groups together, namely, the control, LPS-only, drug-only and LPS+drug groups.

The viability of hPCLS was determined by comparing the ATP content of the treated hPCLS with that of control hPCLS following 24h incubation (Figure 1). The average ATP content of hPCLS following 24h incubation was 7.8 ± 0.7 nmol/mg protein (Table 2). Although the drug concentrations were selected as a kind of lowest observed adverse effect level to cause minimal decrease in ATP content, due to the appreciable variation in the human livers, the selected concentration significantly ($p<0.05$) decreased the ATP content in the PCLS of the liver used for the final studies by 15% for VC, 18% for CZ, 19% for OZ, and 35% for DF after 24h incubation without LPS, whereas no significant decrease was observed for KT, CBZ and TGZ. The lowest observed effect level of VC was much lower in hPCLS (250 μM) compared to mouse PCLS where even 1500 μM did not reduce the ATP content of mPCLS [22]. LPS itself caused slight though significant toxicity in 24h-incubated hPCLS (13% loss of ATP content when compared with the control, n=23). LPS did not enhance the toxicity of DF, CBZ or TGZ in the hPCLS. However, synergistic toxicity was observed when the PCLS were incubated with LPS+KC or LPS+CZ compared to LPS or KC or CZ alone. Their ATP levels reduced to approximately half of the value of the control PCLS. This synergistic toxicity was substantial considering that treatments with CZ or LPS alone only caused slight toxicity and treatment with KC alone did not cause any toxicity at all. In contrast, the respective non-IDILI-associated comparator drugs VC and OZ did not show this phenomenon.

Although the overall effects of LPS+KC and LPS+CZ were significant for the group of livers tested, there was an appreciable variation in the interaction effects of LPS+drug and some human livers did not respond where others responded strongly with synergistic toxicity. For instance, synergistic toxicity was observed in 7 out of 10 human livers cotreated with LPS+KC and in 9 out of 14 human livers cotreated with LPS+CZ. For the other drugs, only 1 out of 5 livers for DF, 3 out of 7 livers for TGZ, and none out of 5 livers for CBZ showed synergistic toxicity in the presence of LPS. The parameter used to distinguish between the responders (synergistic toxicity observed) and non-responders (synergistic toxicity not observed) was based on the ATP data where an individual was considered a responder if the reduction of ATP (compared to the control) caused by the co-incubation of LPS+drug is greater than the ATP reduction caused by LPS alone plus the ATP reduction caused by the drug alone.

The effect of LPS on the toxicity of the drugs was also assessed by histomorphology. The histomorphology data (Figure 2) confirmed the findings from the ATP data. hPCLS from the responder livers that were incubated with the combination of LPS and KC or CZ showed extensive necrotic areas indicated by the loss of nuclei as shown in Figure 2F & 2G, while incubation with LPS alone or the drug alone showed only comparably minor

toxicity. Moreover, in line with the ATP data, DF treatment resulted in quite substantial loss of the viable liver tissue; however there was no difference in the extent of the damage in DF-treated hPCLS in the absence or presence of LPS (Figure 2D & 2H). Similarly, 24h treatments with VC, OZ, CBZ, and TGZ did not show any appreciable difference in damage in the absence or presence of LPS (unpublished results).

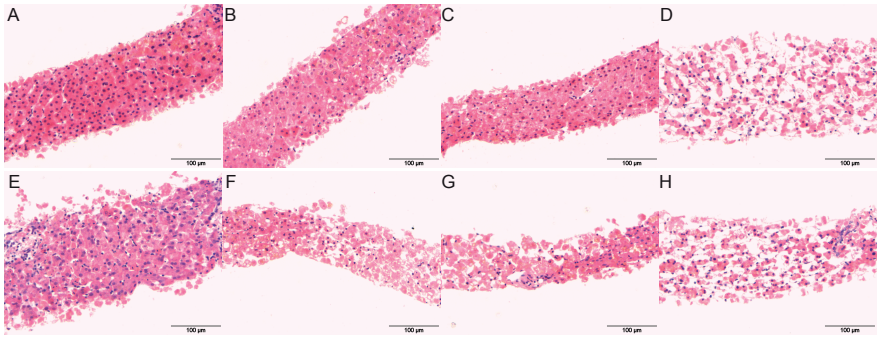

Figure 2. Histomorphology of hPCLS treated respectively with DMSO, KC, CZ, and DF in the absence of LPS (A, B, C, D) and in the presence of LPS (E, F, G, H) for 24h. Five individual experiments were performed with hPCLS. Representative images from a responder liver are shown. The sections were stained by hematoxylin-eosine, bar = 100 µm, magnification 200x.

3.2. Glutathione content of human PCLS

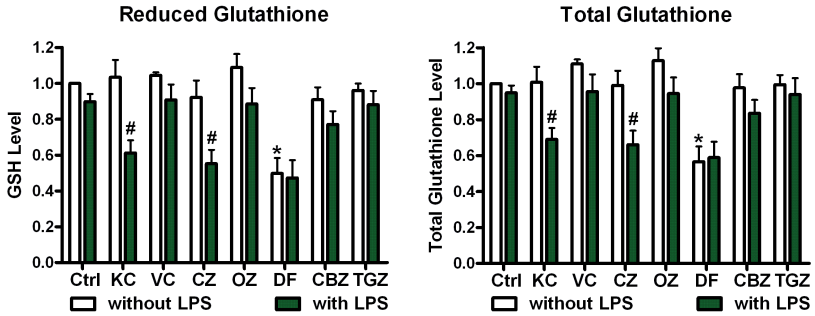

Figure 3. Reduced glutathione (GSH) and total glutathione levels in hPCLS following 24h incubation with various drugs in the absence (open bars) or presence (filled bars) of LPS. The glutathione levels are expressed as relative values to the control hPCLS without LPS-treatment. Data represent the average ± SEM of 5-6 experiments using 3 hPCLS per experiment. *P<0.05 vs control by t-test and #P<0.05 by two-way ANOVA of four different groups together, namely, the control, LPS-only, drug-only and LPS+drug groups.

The reduced and total glutathione levels in hPCLS after 24h incubation are presented in Figure 3. The average GSH and total glutathione content of hPCLS following 24h incubation were 40.9 and 46.5 nmol/mg protein respectively. In contrast to what was found in mouse PCLS where 54% of glutathione was present in the oxidized form, the oxidized glutathione (GSSG) level was low (<10%) in all hPCLS and was not changed after treatment with the drugs and/or LPS. When compared to the control PCLS, none of the drugs alone nor LPS caused any change in GSH or total glutathione levels except for DF that reduced these values to approximately half of the values of the control PCLS. Coincubation with LPS and DF, CBZ, or TGZ did not reduce these values. However, coincubation of either KC or CZ with LPS substantially reduced the levels of both GSH (61% and 55% respectively) and total glutathione (69% and 66% respectively) when compared to the control, LPS-treated and KC- or CZ-treated PCLS. This reduction was observed in 5 out of 6 human livers used in this experiment and was exclusively observed for the two drugs (KC and CZ) that caused synergistic toxicity with LPS but not in the cases of the other drugs in combination with LPS (compare Figures 1 and 3). Since the analysis of the glutathione levels were performed in other livers than the ATP assays due to the limitation of the available tissue, we cannot identify the difference in glutathione levels between responder and non-responder groups based on the ATP data, but the overall effects were significant.

3.3. Metabolism of CZ-treated human PCLS in the absence and presence of LPS

To investigate whether the effect of LPS on the toxicity and the GSH content was caused by increased rate of formation of reactive metabolites, the metabolites of CZ were measured in the media of the PCLS treated with CZ with and without LPS (Figure 4). The liver sources of the hPCLS were separated into two different groups, the responder livers (n=5) and the non-responder livers (n=5) based on the presence of synergistic toxicity of LPS+CZ as described above. The amounts of the metabolites were determined by measuring the peak area, as the absolute amount of each of the metabolite could not be determined due to the unavailability of the references of CZ metabolites, Seven different metabolites of CZ were detected in the media, clozapine-N-oxide (CZ+O) and its reduced form (CZ+O+2H); N-desmethylclozapine (CZ-CH2); glutathione conjugate of CZ (CZ-GSH); cysteine conjugate of CZ (CZ-Cys); cysteine conjugate where chlorine was substituted by GSH and further metabolized to Cys of CZ (CZ-Cys-Cl); and cysteine conjugate of clozapine-N-oxide (CZ-Cys+O). Interestingly, the relative amounts of both CZ-Cys and CZ-GSH in the medium were significantly lower among the responders in hPCLS incubated with LPS when compared to the ones without LPS. Among the non-responders, no difference in the amount of any metabolite was observed in the

absence or presence of LPS. However for the other five metabolites, there was no difference in the amounts produced by hPCLS incubated with or without LPS or between responders and non-responders (unpublished results).

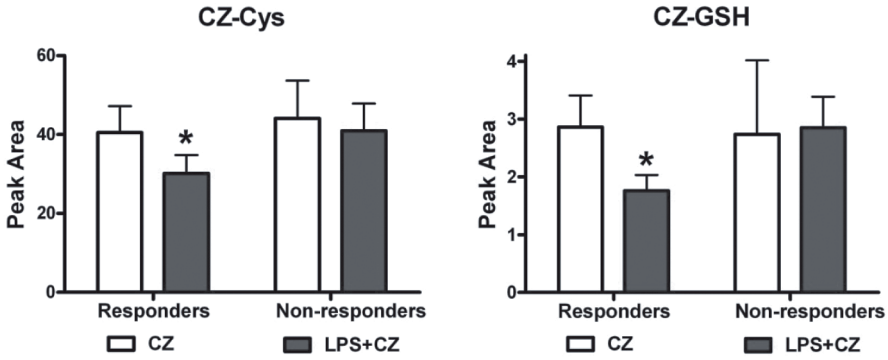

Figure 4. Metabolites produced by hPCLS during 24h incubation with CZ (60 μM) in the absence and presence of LPS. The graphs show the glutathione conjugate (CZ-GSH) and corresponding cysteine conjugate (CZ-Cys) of CZ produced in responders and non-responders by addition of GSH to CZ. Exact structures of these metabolites were not determined. Data were the average of 10 individual human livers consisting of five responders and 5 non-responders using 3 slices per experiment. *P<0.05 for CZ vs. LPS+CZ by t-test.

3.4. LPS-induced release of sICAM-1 cytokines in human PCLS

The release of the soluble form of the chemoattractant sICAM-1 and nine inflammatory cytokines (IL-1β, IL-10, IL-12, IFN-γ, TNF, CCL-3, CCL-5, G-CSF, and GM-CSF) were measured in the media following 24h incubation of the PCLS. LPS treatment of hPCLS induced significant release of sICAM-1 and cytokines into the media (Table 2 and Figure 5). However, IL-10 and IL-12 release could not be quantified because the concentrations were below the detection level and thus no data can be shown for these cytokines. Treatments of PCLS with any of the drugs alone did not result in significant changes in cytokine release when compared to control PCLS. In contrast, all drugs caused changes in several of the LPS-induced release of sICAM-1 and cytokines (Figure 5). sICAM-1 release was increased approximately 3 fold compared to the control by the addition of LPS in hPCLS. Interestingly, all drugs reduced the LPS-induced sICAM-1 release. The effects of the drugs on the release of cytokines varied largely and were drug-specific. DF markedly decreased all the LPS-induced cytokine release, while CBZ decreased all cytokines moderately. On the other hand, TGZ only substantially reduced LPS-induced IFN-γ but did not influence any of the other cytokines. KC, OZ, CBZ and DF significantly decreased the LPS-induced IL-

1β release. All drugs except TGZ lowered the LPS-induced CCL3 release. CZ was the only drug that significantly increased the release of CCL5, while most other drugs decreased it. OZ, DF, and CBZ significantly reduced the LPS-induced release of G-CSF and GM-CSF and KC reduced G-CSF release only, while CZ was the only drug that elevated the release of GM-CSF. Interestingly, KC and CZ, the only two drugs that showed synergistic toxicity with LPS, were also the only drugs that significantly increased the LPS-induced release of TNF (~1.4 and ~3.0 folds respectively). In contrast, the other drugs did not have any influence on its release (OZ and TGZ) or decreased it (VC, CBZ, and DF). Furthermore, KC and CZ were also the only two drugs that did not reduce LPS-induced IFN-γ release, while the other drugs strikingly decreased it. We failed to identify distinct cytokine profiles between responders and non-responders.

Table 2. The levels of ATP, glutathione, ICAM-1, and cytokines following 24h incubation of hPCLS in the absence (control) and presence of LPS. Data are given as mean ± SEM of at least 5 human livers per group. Means of control and LPS-treated groups were compared by paired t-test, *p<0.05, **p<0.01 and ***p<0.001 (LPS-treated hPCLS vs. control hPCLS).

	Control PCLS	LPS-treated PCLS
ATP (nmol/mg protein)	7.8 ± 0.7	6.8 ± 0.7*
Glutathione levels:		
• **Reduced glutathione (nmol/mg protein)**	40.9 ± 7.8	31.9 ± 4.9
• **Total glutathione (nmol/mg protein)**	46.5 ± 9.1	37.9 ± 5.9
sICAM-1 (pg/ml)	698.1 ± 114.7	1926.6 ± 195.2***
Cytokines:		
• **IFN-γ (pg/ml)**	0.6 ± 0.6	53.8 ± 23.2*
• **IL-1β (pg/ml)**	2.6 ± 1.1	111.6 ± 17.0**
• **TNF-α (pg/ml)**	1.1 ± 0.7	105.1 ± 15.9**
• **CCL3 (pg/ml)**	180.3 ± 58.0	2280.2 ± 201.5***
• **CCL5 (pg/ml)**	26.1 ± 4.6	1053.1 ± 209.0**
• **G-CSF (pg/ml)**	17.8 ± 5.4	1708.1 ± 155.8**
• **GM-CSF (pg/ml)**	0.0 ± 0.0	30.0 ± 6.9*

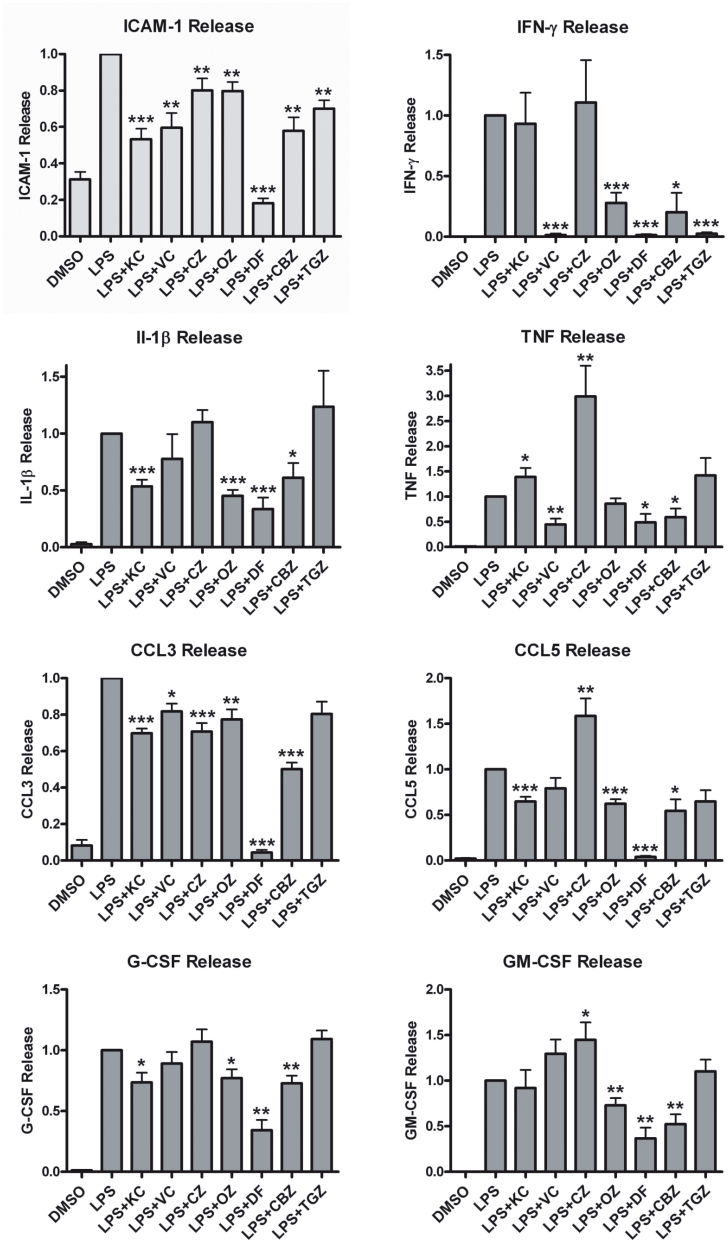

Figure 5.Effect of the various drugs on the LPS-induced release of ICAM-1 and cytokines (IFN-γ, IL-1β, TNF, CCL-3, CCL-5, G-CSF, GM-CSF). The levels of cytokine release are expressed as relative values to the LPS-treated hPCLS. Treatment with any drug alone had no effect in the cytokine release when compared to control hPCLS. Data represent the average ± SEM of 5-22 experiments, using 3 hPCLS per experiment. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. LPS-treated group (t-test).

4. Discussion

Every new drug possesses a potential IDILI liability [46] but the low and sporadic occurrence of IDILI, coupled to the lack of reliable and robust screening methods are two of the reasons that it is challenging to predict and prevent IDILI [47]. Moreover, the mechanisms of action of IDILI in humans have not been fully elucidated. The prediction of IDILI is further severely hampered by the appreciable species differences in the hepatotoxicity between human and animals [3, 48]. Previously, we have used mouse PCLS to study the inflammatory stress hypothesis and successfully identified IDILI-associated drugs apart from their non-IDILI-associated counterparts [22]. In this model, we exploited LPS and drug co-administration in the PCLS as a model for the inflammatory stress hypothesis, which was developed by Roth et al. [15]. Although several studies employing human cell cultures (HepG2 cells, THLE cells and human primary hepatocytes) were published previously as model for IDILI [43, 49-51], the current study is the first attempt to study idiosyncratic drug reactions in human tissue culture.

Five reactive metabolite-producing drugs that are known to cause IDILI in humans (along with two non-IDILI-related comparator drugs), were tested in the absence or presence of LPS in hPCLS system. For a quantitative viability test, we measured ATP levels of hPCLS and histomorphology was used to confirm the ATP data. The selected concentration of OZ (60 μM) was the same as that of CZ, but as OZ is used at a much lower dose in the clinic than CZ, OZ is not associated with DILI in the clinic. The toxicity observed with OZ occurred at approximately ~150*C_{max}. It should be realized that many non-hepatotoxic drugs induce hepatocyte death at concentrations exceeding 100*C_{max} [43]. The observed VC toxicity in hPCLS illustrates a clear species difference as it was not toxic at all in mouse PCLS even at a concentration of 1500 μM [22].

Synergistic hepatotoxicity was observed when hPCLS were co-incubated for 24h with LPS and KC or CZ when compared to the hPCLS treated with LPS or drug alone (Figure 1). The histomorphology data confirmed this finding as extensive necrotic areas can be seen in hPCLS co-incubated with LPS and KC or CZ (Figures 2F and 2G), which were not detected in hPCLS treated with the drugs alone or with LPS alone. This synergistic toxicity related to KC and CZ after 24h incubation was also found in our previous study using mouse PCLS [22].Our results from PCLS are in line with other *in vitro* or *in vivo* results where LPS or cytokines enhanced the toxicity of a number of other IDILI-associated drugs (monocrotaline, ranitidine, trovafloxacin, nefazodone, nimesulide), but not of their corresponding non-toxic comparator drugs, in mice and rats and in cultured cells [19, 20, 43, 50, 52].

The co-exposure of LPS and three other IDILI drugs DF, CBZ, or TGZ did not show synergistic hepatotoxicity in hPCLS after 24h incubation (Figure 1), similar to our previous

findings in mouse PCLS. We have recently shown that in mouse PCLS, synergistic toxicity could be observed in the cases of DF and TGZ after a longer incubation of 48h [22]. Unfortunately, this knowledge was not available at the time these experiments with hPCLS were performed. Future experiments need to show whether this is also the case in human PCLS. It has been shown that different doses of LPS and different exposure times of LPS and drug might be needed to observe the synergistic hepatotoxicity *in vivo* for different drugs [20, 53]. An alternative explanation might be that these drugs cause IDILI via another mechanism than the one proposed in the inflammatory stress hypothesis, such as the danger hypothesis [16, 17] or the pharmacological interaction (p-i) hypothesis [54]. Roth and Ganey argued that a model based on a single hypothesis might not predict adequately all drugs causing IDILI in humans as it is likely that different drugs would exert their toxicity via different mechanisms of action [9].

The development of IDILI involves not only the formation of reactive metabolites but may also involve other factors, e.g. the depletion of GSH or a polymorphism in glutathione transferases [55]. Similar to our previous finding in mouse PCLS [22], significantly lower GSH and total glutathione levels were observed after coincubation of LPS and KC or CZ in hPCLS when compared to incubation without LPS (Figure 3). LPS itself did not cause any reduction in the levels of GSH or total glutathione in control PCLS or PCLS treated with any of the other drug tested. As in mouse PCLS, the reduction in glutathione levels correlated with the synergistic toxicity observed in the hPCLS coincubated with LPS+KC or LPS+CZ (Figures 1, 2, 3). This correlation might signify that less GSH-conjugate was formed due to the lower GSH content in the slices, thus more non-detoxified reactive metabolites were present causing increased liver injury. A decrease in GSH level could also be associated with oxidative stress which is usually accompanied with an increase in GSGSH level [56]. Since we did not find elevated GSGSH level, the role of oxidative stress in the synergistic toxicity of LPS+KC or LPS+CZ is unlikely.

Since idiosyncrasy is defined as individual differences in responses to stimuli [57] and considering the fact that every human is different in terms of genetics, medical history, and environmental exposure, there was obviously a variation in the responses related to toxicity. The synergistic toxicity phenomenon did not occur in the PCLS from all human samples. Our findings were consistent with the fact that IDILI is definitely not observed in all patients taking the same drug. Moreover, this variation was also reported in animal studies, for instance, despite the fact that Brown Norway is a highly inbred strain, the idiosyncratic autoimmunity syndrome only occurred in 50%-80% of the penicillamine-treated rats [58]. Another IDILI model utilizing SOD2+/- mice reported inconsistent liver injury where hepatocellular necrosis occurred in only 4 of 6 TGZ-treated mice [59]. Based

on these observations, we selected five responder and five non-responder livers in the CZ-treated group and re-analyzed the data in these groups separately with respect to CZ metabolism. Although, it was suggested that the reactive metabolites of CZ play a role in agranulocytosis [40], their role in CZ-induced human hepatotoxicity has not been investigated [10]. Formation of glutathione conjugates of CZ (CZ-GSH) have been detected in multiple *in vitro* and *in vivo* systems [60]. Within the responder group, the amounts of the CZ-GSH and CZ-Cys (the breakdown product of CZ-GSH [61]), were significantly lower in the hPCLS incubated in the presence of LPS (Figure 4). In contrast, in the non-responder group, there was no difference in the amounts of these metabolites between the PCLS incubated in the presence or absence of LPS. The lower amounts of CZ-GSH and CZ-Cys in the responder group after coincubation with LPS and CZ can be attributed to the lower GSH level of PCLS (Figure 3), resulting in higher toxicity that might be due to the covalent binding of CZ reactive metabolites to proteins. Out of the six livers used for the determination of GSH content, five were considered responders that demonstrated reduction of GSH after coincubation of LPS+CZ. Further studies are needed to confirm the role of GSH in the synergistic toxicity of LPS and CZ.

It has previously been shown that neutrophils and cytokines play very important roles in the development of IDILI in the inflammatory stress-related models [7, 15, 19, 52]. Although the presence of neutrophils could be detected in the slices by immunohistochemistry (unpublished results), their numbers were too low to allow quantification. The use of PCLS as an *ex vivo* model by definition lacks the accumulation and infiltration of neutrophils after LPS treatment, but as a surrogate marker the release of sICAM-1 to the medium was measured instead. sICAM-1 can be secreted by various cells, such as hepatocytes and endothelial cells. It has been shown that activated neutrophils are recruited via upregulation of ICAM-1 in the endothelial cells [62] and that the increased level of sICAM-1 reflects this upregulation [63]. While LPS treatment significantly induced the release of sICAM-1 by hPCLS, all seven drugs, IDILI and non IDILI-associated, decreased this LPS-induced release of sICAM-1 (Figure 5). Further studies are needed to identify the importance of ICAM-1 and sICAM-1 in relation to the mechanisms of action of IDILI.

LPS has also been shown to elevate the release of inflammatory cytokines in rat [34] mouse [22] and human [36] PCLS. Correspondingly, LPS substantially induced the cytokine release in hPCLS in the current study (Table 2 and Figure 5). To identify possible biomarkers or factors involved in the mechanism of IDILI, we compared the effect of the drugs on the LPS-induced cytokine release. Only the increased TNF was associated with synergistic toxicity of both KC and CZ. None of the measured cytokines could discriminate all 5 IDILI drugs from the 2 non-IDILI drugs. Although the synergistic toxicity was observed after

coincubation with LPS+KC or LPS+CZ in both mouse and human PCLS after 24h, different effects on the LPS-induced cytokine release were observed. In mouse PCLS, the synergistic toxicity of KC and CZ was associated with an elevated LPS-induced release of G-CSF and GM-CSF, but not with elevated TNF release as in hPCLS [22]. In contrast, none of the drugs increased G-CSF release and only CZ increased the release of GM-CSF in hPCLS (Figure 5). In the hPCLS, all drugs except TGZ decreased the LPS-induced CCL3 release and most drugs including OZ also decreased LPS-induced CCL5 release in hPCLS. CZ was the only drug that increased CCL5 release in hPCLS. In mouse PCLS, on the contrary, none of the comparator drugs (VC or OZ) decreased the CCL3 or CCL5 release and none of the drugs increased the release of CCL3 and CCL5 in mouse PCLS [22]. Additionally, none of the drugs increased the LPS-induced release of IL-1β in human PCLS, while in mouse PCLS, three drugs (KC, CZ, and CBZ) increased its release. All drugs except KC and CZ substantially reduced the release of IFN-γ in hPCLS, whereas in mouse PCLS, this was only the case for OZ, DF, and TGZ.

In line with our current results in hPCLS, a critical role of TNF in inflammatory stress-related IDILI mechanism was found previously for trovafloxacin and ranitidine *in vivo*[20, 64, 65], even though it is still controversial whether ranitidine causes IDILI in men [6]. Moreover, the administration of TNF potentiated the hepatotoxicity of trovafloxacin in rats *in vivo*[66] and of diclofenac in HepG2 cells [50]. TNF is also known to activate endothelial cells and to prime neutrophils for activation, leading to the accumulation of neutrophils in tissue [67-69]. These data confirmed the potential role for TNF as a biomarker to identify drugs that cause IDILI in the human liver via an inflammatory stress-related mechanism. We cannot conclude whether G-CSF, CCL3, or IL-1β play a role in the development of synergistic toxicity in hPCLS, but increased GM-CSF and CCL5 appeared to be associated with the synergistic toxicity specific for CZ but not for KC in hPCLS. Furthermore, the roles of IFN-γ in relation to the synergistic toxicity of KC and CZ with LPS need to be further studied.

In conclusion, we have developed an *ex vivo* model utilizing hPCLS to detect inflammatory stress-related IDILI. Two IDILI-associated drugs (KC and CZ) were identified by this model by showing synergistic toxicity with LPS according to the inflammatory stress hypothesis. This synergistic toxicity phenomenon after 24h was accompanied by concomitant GSH depletion and increased LPS-induced TNF release in hPCLS. Therefore, GSH and TNF are potential biomarkers in this hPCLS model to detect IDILI related to inflammatory stress. Moreover, the mechanism of IDILI related to CZ might be explained by depletion of GSH in the liver, resulting in lower GSH- and cysteine-conjugates of CZ. Further research should involve the measurement of these markers in hPCLS after 48h incubation and also the inclusion of more IDILI-related drugs together with their non-IDILI-related

comparator drugs to be tested in hPCLS. Another important conclusion is that there are marked species differences in how the same drug affects LPS-induced cytokine release in mouse and human. This underlines the importance of using human tissue to elucidate the mechanisms of IDILI in humans. Additionally, the use of hPCLS contributes to the reduction and replacement of animals in drug research. Therefore, the hPCLS system may be a prospective *ex vivo* translational screening tool that can be utilized in the preclinical and clinical settings to characterize IDILI at various stages of drug development.

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Summary, Discussion, and Future Directions & Perspectives

6

1. Summary and Discussion

Adverse drug reactions (ADRs) are a serious problem for public health and an obstruction to drug development and clinical use after marketing. ADRs affect millions of people and can result in hospitalization and even death. Among all ADR cases, the liver is the most frequently affected organ due to its high exposure and capacity to metabolize drugs. Drug-induced liver injury (DILI) is the number one cause of acute liver failure cases in the U.S. and has also been the top reason of drug withdrawal from the market [1-4].

As introduced in **chapter 1**, DILI can be categorized into intrinsic (type A) vs. idiosyncratic (type B) DILI. Intrinsic DILI usually exhibits dose and time dependency and can be detected during pre-clinical or clinical trials. Overdose of the intrinsic hepatotoxin paracetamol or acetaminophen (APAP), is responsible for most of the cases of liver failure among any other drugs [3]. A reactive metabolite of APAP, *N*-acetyl-*p*-benzo-quinone imine (NAPQI), is thought to be responsible for APAP-related hepatotoxicity. On the other hand, idiosyncratic DILI occurs unpredictably in a small minority of the people taking the same drug (less than 1 in 1000 people), is not related to the pharmacological effects of the drug and does not have an obvious dependence to dose or time. While intrinsic DILI normally occurs during drug overdose, idiosyncratic DILI can occur when patients taking normal therapeutical doses of a drug and is responsible for approximately 13% of acute liver failure cases in the US [3]. Several hypotheses have been described to explain the mechanisms of action of idiosyncratic DILI and many of these hypotheses involve the metabolism of drugs into reactive metabolites [5, 6]. One hypothesis is called the 'inflammatory stress' hypothesis because it states that the presence of a concurrent inflammatory reaction can interact with drug therapy to precipitate idiosyncratic DILI. This modest inflammatory stress can lower the threshold for drug hepatotoxicity, thereby shrinking the therapeutic window resulting in a toxic response at an otherwise safe dose of the drug. Alternatively, a drug might also augment a mild inflammatory reaction rendering it injurious [7, 8]. Several in vivo and in vitro models based on this inflammatory stress hypothesis have been developed involving coexposure with drugs associated with human idiosyncratic DILI and an inflammagen, such as lipopolysaccharide (LPS), to produce synergistic (or idiosyncratic) hepatotoxicity. The current thesis focused on the development of a potential *ex vivo* model utilizing precision-cut liver slices (PCLS), in which all liver cell types are present possessing normal liver tissue architecture, to study the species differences in intrinsic (chapters 2 & 3) and idiosyncratic (chapters 4 & 5) DILI. There are several advantages of utilizing PCLS over other *in vitro* systems. PCLS are superior to primary hepatocytes and HepG2 cells as these systems are lacking the other liver cell types and at least in the case of HepG2 cells, they also possess inadequate biotransformation capacity [9, 10]. Co-culturing hepatocytes

and other cells was used as a model but this is a quite complex method as it is difficult to assess the correct ratio of the different cell types and to establish correct cell to cell contacts. Additionally, in the idiosyncratic DILI model involving inflammatory reactions, cytokines are suggested to play a role. Even though it is possible to add cytokines to the hepatocyte cultures, PCLS supposedly give more representative levels and mixtures of cytokines produced by inflammatory reactions (such as ones induced by LPS) similar to the situations in the real liver.

Species difference in the hepatotoxicity of APAP has been previously investigated in primary hepatocytes [11]. In **chapter 2**, similar findings were confirmed in PCLS, in which mouse was the most sensitive species to APAP toxicity, followed by rat, and then human. In studies on the mechanism of toxicity of a xenobiotic, a non-toxic analog with a pharmacological activity similar to that of the toxic compound is frequently taken as control to distinguish pharmacological from toxicological effects. Therefore, the toxicity of N-acetyl-meta-aminophenol (AMAP), a regioisomer of APAP with similar analgesic and antipyretic properties, was also tested in PCLS from these species. AMAP has been widely considered as a non-toxic isomer of APAP, even though AMAP had only been studied in mice and hamsters. Although AMAP indeed did not cause hepatotoxicity at concentrations where APAP was toxic in mouse PCLS, surprisingly, AMAP was equally toxic as or even more toxic than APAP at all concentrations tested in both rat and human PCLS based on the ATP level and histomorphology of the slices. Thus, AMAP is potentially toxic in men. Interestingly, no glutathione conjugate or S-containing metabolites of the GSH conjugate of AMAP were detected in all three species. Moreover, PCLS from mouse, the least sensitive species to AMAP toxicity, produced ten times more hydroquinone metabolites (the assumed proximate reactive metabolites) of AMAP compared to rat or human PCLS. This suggested that the toxicity of AMAP in rat and human is not caused by the alleged proximate reactive hydroquinone metabolites. Conclusively, there were marked species differences in the toxicity and metabolism of APAP and AMAP.

These findings indicate the importance of using a human test system when selecting non-toxic analogs of drugs. We also showed that human and animal PCLS can be a suitable and straightforward method to test species differences in hepatotoxicity.

To investigate the potency to use excreted proteins as biomarker for DILI, the profiles of proteins excreted into the medium of PCLS as a result of drug-induced injury were analyzed in **chapter 3** and compared to urine profiles in mice treated with APAP. The toxicity of AMAP in rat and human PCLS was confirmed as the profiles of the proteins in the medium of AMAP-treated PCLS were very similar to the profiles of APAP-treated PCLS and differed from the controls. However, in mouse PCLS, the protein profiles of

APAP-treated and AMAP-treated PCLS medium were substantially different, which is in line with the observation that the applied concentration of AMAP was not toxic in mouse PCLS. Furthermore, protein profiles of mouse PCLS medium were shown to resemble *in vivo* mouse urinary profiles in the case of APAP-induced liver injury. Protein identification revealed the same key proteins in the medium of APAP-treated mouse PCLS as in the urine of APAP treated mice. Treatments of both APAP and AMAP in rat and human PCLS as well as treatment of APAP only in mouse PCLS resulted in a marked decrease in hepcidin concentration in the PCLS medium. PCLS from these three species treated with another hepatotoxin, diclofenac (DF), also exhibited a decreased hepcidin concentration in the medium, while treatment with lipopolysaccharide (LPS) significantly increased it. These findings correlated well with previous in vivo data where APAP treatment decreased the hepcidin level in the plasma and livers of mice with APAP-induced liver injury, as a result of oxidative stress (van Swelm et al., 2012) and that LPS was known to be a strong inducer of hepcidin [12].

In conclusion, the use of PCLS may be useful to identify urinary proteins as liver-derived proteins. Moreover, protein profiling can be a promising and non-invasive method for biomarker identification *in vitro/ex vivo* (in medium) or *in vivo* (in urine) and hepcidin might serve as a potential novel *in vitro/ex vivo* biomarker for DILI.

It was hypothesized that APAP-induced liver injury occurs by two phases, a metabolic phase (involving GSH depletion and protein adducts) and an oxidative phase (involving oxidative stress and mitochondrial permeability transition (MPT)) [13, 14]. MPT is an abrupt increase in the permeability of the inner mitochondrial membrane to ions and small molecular weight solutes. MPT is associated with increased oxidative stress as well as decreased ATP synthesis and it causes superoxide anion release from the mitochondria, which is lethal for the cell [13, 15]. Cyclosporine A (CsA) binds to cyclophilin D in the MPT pore and has been shown to be a specific and potent inhibitor of MPT [15, 16]. Cyclosporine A was also used to decrease oxidative stress and attenuate APAP-induced hepatotoxicity in mice *in vivo* and *in vitro*[14, 17]. To investigate whether the unexpected AMAP toxicity in rat and human PCLS is associated with MPT, we coincubated PCLS with AMAP (5 mM) and CsA for 24h. While 2.5 μM CsA already slightly reduced the viability of rat PCLS by ~20%, in human PCLS, CsA was not toxic up to 5 μM. Therefore, the effects of CsA were studied at concentrations of 5 and 2.5 μM in rat and human PCLS respectively. Surprisingly, instead of reducing the toxicity of AMAP, CsA significantly enhanced the toxicity of AMAP in both rat and human PCLS, as coincubation with AMAP and CsA decreased the ATP levels of the PCLS to approximately half of the values of the AMAP-treated rat or human PCLS. It was previously reported that the addition of CsA as a chemosensitizer could enhance

the toxic adverse reactions of doxorubicin, such as nephrotoxicity, in rats. However, under circumstances where doxorubicin caused only a low level of toxicity in the liver of these rats, the combined treatment of doxorubicin and CsA did not actually enhance this hepatotoxicity [18]. More recently, chronic administration of CsA by itself was shown to be capable of inducing hepatotoxicity in rats and guinea pigs by generating reactive oxygen species (ROS) [19-22].

In conclusion, our results do not provide evidence for an involvement of the MPT in AMAP-induced liver injury and AMAP may interact with CsA producing synergistic hepatotoxicity as observed in Figure 1. However, further research is needed to elucidate the mechanisms of hepatotoxicity involving this interaction between AMAP and CsA.

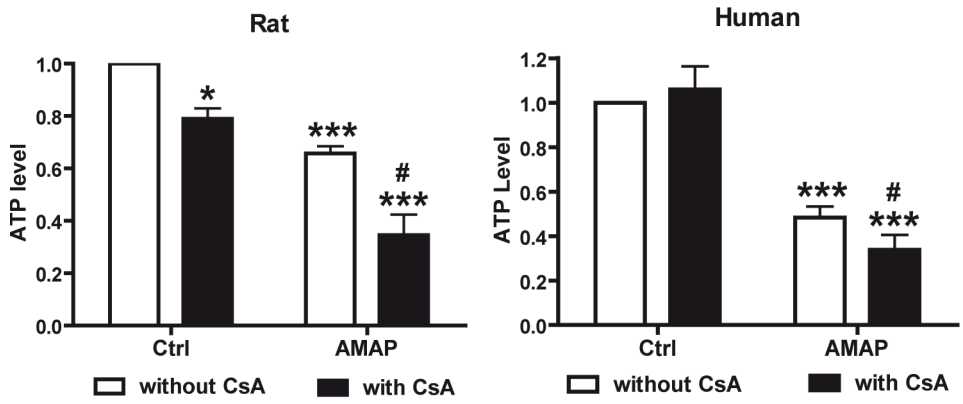

Figure 1. Viability of PCLS indicated by the ATP content after 24h incubation with AMAP (5 mM) in the absence (open bars) or presence (black bars) of CsA (5 μM in rat or 2.5 μM in human). ATP content is expressed as relative values to the respective control PCLS without CsA-treatment. Data represent the average ± SEM of 4 (rat) or 6 (human) experiments using 3 PCLS per experiment. *P<0.05 or ***P<0.001 vs. control PCLS, #P<0.05 vs. AMAP-treated PCLS by paired t-test.

Based on these results and other previously-performed experiments [23, 24], PCLS appear to be a promising tool to identify drugs that potentially cause intrinsic DILI and to study the mechanisms behind the toxicity.

Chapter 4 and **chapter 5** focused on the development of an *ex vivo* model using PCLS to detect idiosyncratic DILI in mouse and human PCLS. We developed our model based on the inflammatory stress hypothesis proposed by Roth et al. involving the simultaneous exposure to idiosyncratic DILI-inducing drug and LPS as an inflammagen [7]. We

investigated both the effects of LPS on the toxicity of the drugs and the effects of the drugs on the LPS-induced inflammatory reactions. Five drugs that produce reactive metabolites and are known to cause idiosyncratic DILI in humans were selected to be tested in our model. These drugs were ketoconazole (KC), clozapine (CZ), diclofenac (DF), troglitazone (TGZ), and carbamazepine (CBZ). Following 24h incubation, both mouse and human PCLS coincubated with LPS and KC or CZ exhibited synergistic toxicity based on ATP levels, histomorphology, and (only in mouse) leakage of lactate dehydrogenase (LDH). Voriconazole (VC), the comparator drug for KC that is not known to show idiosyncratic DILI, was not toxic in mouse PCLS or in rats in vivo [25], but it causes dose-dependent non-idiosyncratic hepatotoxicity that occurs in approximately 51% of patients [26, 27]. Despite VC-induced toxicity, coincubation with LPS and VC, did not cause synergistic toxicity. Likewise, incubation of olanzapine (OZ), the comparator drug of CZ, and LPS also did not cause synergistic toxicity. This synergistic toxicity phenomenon was accompanied by a concomitant depletion of glutathione (GSH). The other 3 drugs that cause idiosyncratic DILI in humans, DF, TGZ, and CBZ, did not show synergistic toxicity with LPS after 24h incubation in both mouse and human PCLS and no depletion of GSH. We also studied the effect on the LPS-induced cytokine production as cytokines have been suggested to be involved in the mechanisms of idiosyncratic toxicity [28] and moreover, we wanted to investigate whether they could be used as potential biomarkers for idiosyncratic DILI. None of the drugs alone significantly induced changes in the release of any cytokine, while LPS significantly elevated the release of the following cytokines: interferon gamma (IFN-γ), interleukin-1beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor (TNF), CC chemokine ligand 3(CCL3), CC chemokine ligand 5(CCL5), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Our strategy was to search for common phenomena in the LPS induced synergistic toxicity of KC and CZ. In mouse PCLS, KC and CZ were the only two drugs that increased the LPS-induced release of G-CSF and GM-CSF. On the other hand, this was not the case in human PCLS where TNF was the only cytokine that was further elevated by KC and CZ and not by the other drugs after 24h incubation. Our finding on TNF in the human PCLS was in line with others who reported that TNF also plays a critical role in inflammatory stress-related idiosyncratic DILI mechanism of aflatoxin B1, trovafloxacin, and ranitidine in rats and mice *in vivo*[29-32].

These data suggest that TNF as well as GSH can be used as predictive biomarkers of inflammatory stress-related idiosyncratic DILI in human PCLS, and potentially in other *in vitro*/*ex vivo* systems. Furthermore, GSH can also serve as a translational biomarker in mice *in vitro*, *ex vivo*, and maybe even *in vivo*. Despite similar findings in the synergistic

toxicity phenomenon related to LPS+KC and LPS+CZ in both mouse and human PCLS, there were marked species differences in how these drugs affect LPS-induced release of cytokines, suggesting different mechanisms of idiosyncratic hepatotoxicity might be involved between mouse and human.

Initially, we observed that there was a concomitant increase in IL-1β in the cases of synergistic hepatotoxicity related to LPS+KC and LPS+CZ in mouse PCLS. Therefore we investigated whether IL-1β could be the cause of this synergistic toxicity. However, the use of the pro-inflammatory cytokine interleukin IL-1β instead of LPS did not potentiate the hepatotoxicity of KC or CZ in mouse PCLS. In addition, the use of neutralizing antibody anti-IL1β also did not attenuate the synergistic toxicity caused by coincubation of mouse PCLS with LPS and KC or CZ (unpublished results). These observations suggest that more cytokines and other inflammatory mediators are probably involved in the synergistic toxicity mechanisms here. This is in line with the findings of Cosgrove *et al.* who showed that the incubation of HepG2 cells and primary hepatocytes with a mix of several pro-inflammatory cytokines when compared to one individual cytokine produced the most significant synergistic toxicity of several idiosyncratic drugs tested [28].

It was quite unexpected to find synergistic toxicity only in 2 out of 5 idiosyncratic drugs as they are all known to cause IDILI in man. Hence, in **chapter 4**, we investigated if there is any correlation between incubation time and of the observation of synergistic toxicity in mouse PCLS. We found that two of the idiosyncratic DILI-related drugs that did not cause synergistic toxicity with LPS after 24h incubation, namely DF and TGZ, demonstrated synergistic toxicity with LPS following 48h incubation of mouse PCLS based on the ATP contents. However, even after 48h, LPS still did not enhance the toxicity of CBZ in mouse PCLS. It cannot be excluded that different concentrations of LPS or CBZ and different exposure times between them are needed to observe the synergistic hepatotoxicity for CBZ. An alternative explanation might be that CBZ causes idiosyncratic DILI via another mechanism than the one proposed in the inflammatory stress hypothesis, such as the danger hypothesis [33, 34] or the pharmacological interaction (PI) hypothesis [35]. Future investigations should focus on the measurement of the LPS-induced release of cytokines after 48h incubation and compare it with data from the 24h incubation.

In **chapter 5**, we introduced the terms 'responders' (synergistic toxicity observed) and 'non-responders' (synergistic toxicity not observed) among human individuals. Although the overall effects of LPS+KC and LPS+CZ were significant for the total group of livers tested, there was an appreciable variation in the interaction effects of LPS+drug implying that some human livers did not respond where others responded strongly with synergistic toxicity. For instance, synergistic toxicity was observed in 7 out of 10 human livers (70%

responders) cotreated with LPS+KC and in 9 out of 14 human livers (64% responders) cotreated with LPS+CZ. On the other hand, only 1 out of 8 mice was considered as a non responder. Interestingly, we found that the relative amounts of both the glutathione conjugate (CZ-GSH) and the cysteine conjugate (CZ-Cys) of CZ were significantly lower among the responders in hPCLS incubated with LPS when compared to the ones without LPS. Among the non-responders, no difference in the amount of any metabolite was observed in the absence or presence of LPS. The lower amounts of CZ-SG and CZ-Cys in the responder group after coincubation with LPS and CZ can be attributed to the lower GSH level of this group of PCLS, resulting in the higher amount of non-detoxified reactive metabolites of CZ leading to higher toxicity that might be due to the covalent binding of these metabolites to proteins. This variance in the metabolic profiles of different people could be attributed to genetic polymorphisms in enzymes responsible for the production of reactive metabolites. Also polymorphisms in glutathione S-transferase were suggested to have the potential to increase the susceptibility to idiosyncratic DILI [36]. It would be interesting to further investigate these factors.

Neutrophils were found to be a mediator of injury in LPS-potentiated hepatotoxicity in rats *in vivo*, induced by aflatoxin B1, monocrotaline, and trovafloxacin [37-39] and are involved in induction of liver injury by hepatotoxic doses of LPS [40]. During activation, neutrophils release various cytotoxic factors, including ROS and proteases [41]. It was shown that neutrophil proteases might contribute to hepatocellular injury by mechanisms independent of direct hepatocyte killing [42]. One disadvantage related to the use of PCLS to predict idiosyncratic DILI related to inflammatory stress hypothesis is the absence of neutrophil accumulation and infiltration from the circulation into the liver after LPS treatment. However, it cannot be excluded that the neutrophils present in the liver tissue during harvesting may play a role in the synergistic toxicity in PCLS.

In an attempt to find more sensitive biomarkers that can be used to predict idiosyncratic DILI, we investigated the roles of keratin-18 (K18) and high mobility group box-1 (HMGB1) proteins in our LPS-potentiated hepatotoxicity model in PCLS. K18 and HMGB1 are two recently-developed biomarkers for liver injury that have been previously reported as circulating mechanistic indicators of cell death mode in animal models [43-45] and in clinical studies [46]. There are two forms of K18, namely, full length K18 (FL-K18) and caspase-cleaved K18 (C-K18). FL-K18 and HMGB1 are released passively during necrotic cell death, while C-K18 is generated during apoptosis [47, 48]. The levels of both HMGB1 and the two forms of K18 were significantly elevated during APAP-induced liver injury in mice, in which the elevation of HMGB1 in mouse serum was detected earlier than of alanine aminotransferase (ALT) [43-45], which is one of the most commonly used biomarkers for

liver injury. Subsequently, we measured the release of these three proteins in the medium samples of mouse and human PCLS, which were coincubated with idiosyncratic DILI-associated drugs and LPS for 24h in collaboration with the group of Prof. Kevin Park at University of Liverpool (unpublished results). The leakage of ALT of the same medium samples was also measured for comparison between ALT and HMGB1 or FL-K18. We did not see an extra release of one of these markers due to LPS coincubation. Unexpectedly, we found a perfect correlation between the obtained values of ALT and either K18 or HMGB1, irrespective of the drug treatment and the presence or absence of LPS in mouse PCLS (Figure 2). This finding suggests that these two new biomarkers are not superior in PCLS when compared to ALT leakage.

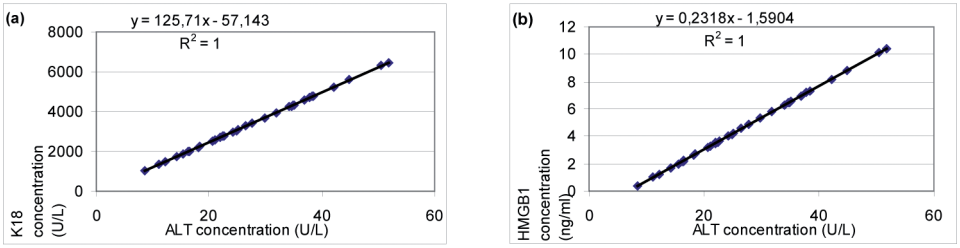

Figure 2. The absolute values of ALT plotted against the absolute values of (a) full-length K18 or (b) HMGB1 in mouse PCLS. These values were measured under various conditions in the absence or presence of different drug and/or LPS.

Furthermore, transcriptomics can be a powerful tool to improve the ability to predict, detect, and characterize idiosyncratic DILI. Toxicogenomics is widely used in drug discovery and development to screen candidate drugs and study their adverse effects and toxic risks. Toxicogenomics allows for the elucidation of molecular mechanisms of toxicity and the detection of biomarkers for toxicity [24]. We performed DNA microarray studies in human PCLS incubated with DF, KC, CZ, and OZ in the absence and presence of LPS for 24h. With the aim to find new biomarkers that can detect and predict idiosyncratic DILI, we applied Affymetrix DNA array expression data analysis to find specific genes that are significantly upregulated or downregulated only in the combination treatment (LPS+idiosyncratic drug) sample and not in the controls, nor in samples treated with drug alone or LPS alone, nor in samples treated with the non-toxic comparator drug in the presence of LPS. The genes that are only specifically regulated in the PCLS coincubated with LPS and idiosyncratic drugs and not regulated in negative comparator drugs can potentially be new biomarkers for idiosyncratic DILI and shed light on its mechanism.

These analyses are currently ongoing. However, a preliminary analysis indicated that most changes in gene expression were in the PCLS cotreated with LPS+KC and LPS+CZ (Figure 3). These two treatment groups also showed the highest numbers of overlapping genes that are significantly changed. There were few overlapping genes between LPS+CZ and LPS+OZ groups and LPS+DF cotreatment appeared to show a very distinct gene expression profile when compared to the other drugs. These findings may suggest some common idiosyncratic hepatotoxicity pathways between KC and CZ and the 49 upregulated common genes as well as 25 downregulated common genes may serve as biomarkers for idiosyncratic DILI in PCLS. Further analyses of these microarray data need to be performed to elucidate more in-depth mechanisms and to find biomarkers related to idiosyncratic DILI.

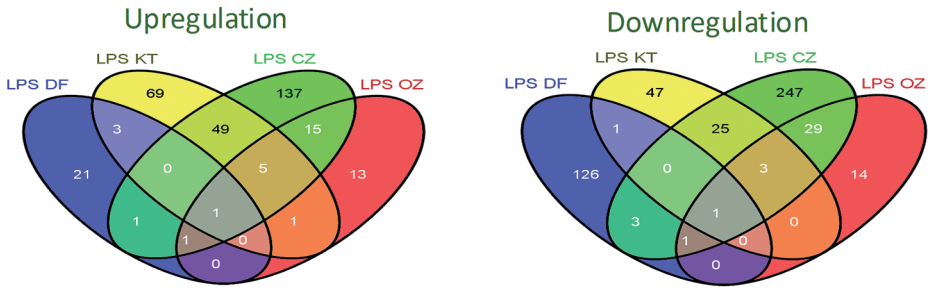

Figure 3. The number of genes regulated only in the 'LPS+drug' groups (FC>2 against control, FDR<0.01) and not regulated in 'LPS only' or 'drug only' groups (FC 'LPS+drug' > 1.5 * FC 'LPS only' or 'drug only').

2. Concluding Remarks

We have developed an *ex vivo* model utilizing rat, mouse, and human PCLS that is capable to detect intrinsic DILI (APAP-induced) and idiosyncratic DILI related to inflammatory stress hypothesis. This *ex vivo* PCLS model is considered a higher throughput model when compared to *in vivo* models and at the same time it is also a more representative model than other *in vitro* models as all liver cell types are present in their natural environment in PCLS, which is important because of the possibility to identify the cytokines and the metabolites produced by the PCLS. Moreover, the possible interindividual differences observed in PCLS are also important to identify sensitive individuals. Another significant advantage is that PCLS can be a robust translational model with the possibility of using liver tissue from animals and humans and compare their findings. Our studies have identified not only significant differences between rat, mouse and human PCLS in their sensitivity for the toxicity of drugs like APAP, AMAP and VC to mention a few, but we also detected prominent species differences in the cytokine release upon LPS challenge and LPS+drug

treatment. We identified hepcidin as a translational biomarker for intrinsic DILI in PCLS and it can also be further investigated as an in vivo DILI biomarker in humans. GSH can be another prospective translational biomarker that can be measured in *in vitro* and *ex vivo* systems as well as in animals *in vivo*. In addition, TNF is considered as a budding *in vitro*/*ex vivo* biomarker for inflammatory stress-related idiosyncratic DILI in humans but not in mice. The species differences in toxicity, metabolism, and the effects on LPS-induced cytokine release underline the importance of human experiments in addition to animal experiments to elucidate the mechanisms of intrinsic and idiosyncratic DILI in humans. Moreover, the use of PCLS contributes to the reduction of the number of animals used in research as a liver from an individual animal can generate many PCLS (~40 from a mouse liver and ~200 from a rat liver) than can be used to test multiple treatment conditions. Similarly, the use of human PCLS may also contribute to the reduction and eventually replacement of animal experiments in toxicology.

3. Future Directions and Perspectives

To follow up and advance the studies initiated in this thesis, there are several possibilities for future research. Generally speaking, larger numbers of idiosyncratic DILI-related drugs together with their negative comparator drugs may be included and tested in PCLS because it is very likely that different drugs would exert their toxicity via different mechanisms of action [49]. As described in chapter 4, prolonging the incubation time from 24h to 48h may identify more drugs as idiosyncratic DILI inducing drugs. The 48h experiments were only performed in mouse PCLS, thus future experiments should elucidate if longer incubation of human PCLS will also show synergistic toxicity of LPS with either DF, TGZ, CBZ, or any other drugs that are associated with idiosyncratic DILI with concomitant decrease in GSH and changes in LPS-induced cytokine release. Additionally, metabolite profiling, with particular interest in the GSH conjugation, of KC and other idiosyncratic drugs that show synergistic toxicity should be performed to identify any differences in the metabolism profiles between responders and non-responders in the absence or presence of LPS, combined with analyzing the polymorphisms in GST or phase 1 enzymes involved in bioactivation of the drugs. The new information, combined with the current metabolism data of CZ, will give us more information on the role of reactive metabolites in idiosyncratic DILI in PCLS.

The gene microarray analysis is a promising powerful tool to study the mechanisms of intrinsic and idiosyncratic DILI. Pathway analysis can elucidate the specific biological or toxicological pathways involved in the mechanisms of idiosyncratic DILI in PCLS. All these combined data can contribute to establishing PCLS as a powerful *ex vivo* screening

tool for idiosyncratic DILI.

Ultimately, thorough screening of drug candidates is necessary to correctly choose the relatively safest drug candidates and avoid those with high potential of causing intrinsic and idiosyncratic DILI. The analyses of the production of reactive metabolites, levels of hepcidin, and the levels of GSH, TNF or other cytokines after cotreatment with LPS was demonstrated to be promising here.

In conclusion, the PCLS system may be a robust and promising *ex vivo* translational screening tool that can be utilized in the preclinical settings to identify and characterize human-specific intrinsic and idiosyncratic DILI at various stages of drug discovery and development.

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Samenvatting

Schadelijke bijwerkingen van medicijnen (in het engels aangeduid als Adverse Drug Reactions, ADR's) vormen een ernstig probleem voor de volksgezondheid en een belemmering voor de ontwikkeling van geneesmiddelen en het klinische gebruik na marketing. ADR's treffen miljoenen mensen en kunnen leiden tot ziekenhuisopname en zelfs de dood. De lever is het orgaan dat het meest te lijden heeft van ADRs vanwege de hoge blootstelling aan geneesmiddelen en het vermogen om ze te metaboliseren tot schadelijke verbindingen. Geneesmiddel-geïnduceerde leverbeschadiging (drug-induced liver injury, DILI) is de belangrijkste oorzaak van acuut leverfalen en het is ook een van de belangrijkste redenen waarom geneesmiddelen uit de handel genomen worden.

Zoals beschreven in **hoofdstuk 1** kan DILI worden onderverdeeld in intrinsieke (type A) en idiosyncratische (type B) DILI. Intrinsieke DILI vertoont meestal een dosis- en tijdsafhankelijkheid en het wordt vaak al gedetecteerd tijdens de pre-klinische of vroege klinische testen. De meeste gevallen van geneesmiddel-geïnduceerde leverfalen worden veroorzaakt door overdosering van het intrinsiek levertoxische paracetamol (APAP). De verantwoordelijkheid voor de levertoxiciteit van paracetamol wordt toegeschreven aan een reactieve metaboliet van APAP, N-acetyl-p-benzo-quinone imine (NAPQI). Idiosyncratische DILI daarentegen komt voor in een zeer kleine minderheid (minder dan 1 op de 1000) van de mensen die een normale therapeutische dosering van het geneesmiddel nemen. Het toxische effect is niet of nauwelijks voorspelbaar tijdens preklinische en klinische studies, en is niet gerelateerd aan de farmacologische effecten van het geneesmiddel. Terwijl intrinsieke DILI meestal optreedt na een overdosis van het medicijn, kan idiosyncratische DILI optreden bij patiënten die een normale therapeutische dosis van een geneesmiddel krijgen. Verscheidene hypothesen zijn beschreven om de werkingsmechanismen van idiosyncratische DILI te verklaren en veel van deze hypothesen gaan uit van het ontstaan van reactieve metabolieten van geneesmiddelen. Eén van die hypotheses wordt de 'inflammatoire stress' hypothese genoemd, omdat die is gebaseerd op de veronderstelling dat een gelijktijdig optreden van een ontstekingsreactie tijdens medicijngebruik kan leiden tot idiosyncratische DILI. Deze inflammatoire stress kan de drempel voor levertoxiciteit door een geneesmiddel verlagen, waardoor het therapeutische venster kleiner wordt, wat uiteindelijk resulteert in een toxische respons op een normaal gesproken veilige dosis van het medicijn. Ook kan een geneesmiddel een milde ontstekingsreactie versterken waardoor er weefselschade ontstaat. Verscheidene in vivo en in vitro modellen zijn op basis van deze inflammatoire stress hypothese ontwikkeld, waaronder gelijktijdige blootstelling aan geneesmiddelen met een ontstekingsbevorderende stof, zoals lipopolysaccharide (LPS), om synergistische (of idiosyncratische) levertoxiciteit te produceren. Dit proefschrift is

gericht op de ontwikkeling van een mogelijk ex vivo model dat gebruik maakt van precies gesneden leverplakjes (PCLS). Dit weefselpreparaat bezit nog de normale leverarchitectuur, waarbij alle levercellen aanwezig zijn in hun fysiologische verhouding en in hun normale matrix. PCLS gemaakt van lever van verschillende proefdierspecies en van de mens zijn gebruikt om verschillen tussen mens en proefdier in intrinsieke (hoofdstukken 2 en 3) en idiosyncratische (hoofdstukken 4 en 5) DILI te bestuderen.

De levertoxiciteit van APAP is eerder onderzocht in verschillende diersoorten met behulp van primaire hepatocyten. In **hoofdstuk 2** werden soortgelijke bevindingen bevestigd in PCLS, waarin de muis de meest gevoelige soort bleek te zijn voor APAP toxiciteit, gevolgd door de rat en daarna de mens. In onderzoek naar het mechanisme van de toxiciteit van een xenobioticum (lichaamsvreemde stof) wordt vaak een niet-toxisch analogon met een vergelijkbare farmacologische activiteit gebruikt als controle om farmacologische van toxicologische effecten te onderscheiden. Daarom werd de toxiciteit van N-acetyl-meta-aminofenol (AMAP), een regioisomeer van APAP met vergelijkbare analgetische en antipyretische eigenschappen, ook getest in PCLS van deze soorten. AMAP wordt algemeen beschouwd als een niet-toxische isomeer van APAP, ofschoon AMAP alleen is onderzocht in muizen en hamsters. Hoewel AMAP inderdaad geen levertoxiciteit veroorzaakte bij concentraties waar APAP toxisch was in PCLS van de muis, was AMAP verrassend genoeg even toxisch of zelfs toxischer dan APAP bij alle concentraties die getest werden in zowel PCLS van de rat als de mens op basis van het ATP-niveau en de histomorfologie van de leverplakjes. Hieruit kan worden geconcludeerd dat AMAP potentieel toxisch is in de mens, en dus niet als niet-toxisch analogon gebruikt kan worden in het onderzoek. Interessant daarbij is dat in geen van de drie onderzochte species het glutathionconjugaat of S-bevattende metabolieten van het glutathionconjugaat van AMAP werden gedetecteerd. Bovendien produceerden PCLS van de muis, de minst gevoelige soort voor AMAP toxiciteit, tien keer meer hydroquinon metabolieten (de metaboliet waaruit de veronderstelde reactieve metaboliet wordt gevormd) van AMAP dan ratten of humane PCLS. Dit suggereerde dat de toxiciteit van AMAP in ratten en mensen niet veroorzaakt wordt door de veronderstelde reactieve hydroquinon metabolieten. Concluderend, er werd een duidelijk verschil in de toxiciteit en het metabolisme van APAP en AMAP tussen soorten gevonden. Deze bevindingen wijzen op het belang van een testsysteem met menselijk weefsel bij het selecteren van niet-toxische analogen van medicijnen. We toonden ook aan dat het gebruik van PCLS van zowel mens als dier een geschikte en eenvoudige methode kan zijn om soortverschillen in levertoxiciteit te testen.

Om te onderzoeken of uitgescheiden eiwitten als biomarker voor DILI gebruikt kunnen worden, werden de profielen van eiwitten die uitgescheiden waren in het medium van de

PCLS als gevolg van geneesmiddel-geïnduceerde schade in **hoofdstuk 3** onderzocht en vergeleken met eiwitprofielen in de urine van muizen behandeld met APAP. De toxiciteit van AMAP in PCLS van rat en mens werd bevestigd doordat de profielen van de eiwitten in het medium van AMAP behandelde PCLS vergelijkbaar waren met de profielen van APAP behandelde PCLS en verschilden van de controles. Zoals verwacht waren de eiwitprofielen van het medium van APAP-behandelde muizen PCLS wezenlijk verschillend van die van AMAP-behandelde PCLS, dit is in overeenstemming met de waarneming dat de toegepaste concentratie van AMAP niet toxisch was in PCLS van muizen. Bovendien leken de eiwitprofielen van het medium van muizen PCLS blootgesteld aan APAP op de urine profielen van muizen *in vivo* met APAP geïnduceerde leverbeschadiging. Eiwitidentificatie onthulde dat in het medium van APAP behandelde muizen PCLS in belangrijke mate dezelfde eiwitten zaten als in de urine van APAP behandelde muizen. Behandelingen van ratten en humane PCLS met zowel APAP en AMAP en behandeling van muizen PCLS met alleen APAP in resulteerde in een sterke afname in de hepcidine concentratie in het PCLS medium. PCLS van deze drie soorten behandeld met een ander hepatotoxische stof, diclofenac (DF), vertoonde eveneens een verminderde hepcidine concentratie in het medium, terwijl behandeling met lipopolysaccharide (LPS) de concentratie significant deed toenemen. Deze bevindingen correleerden goed met eerdere *in vivo* data waar APAP het hepcidine niveau in het plasma en de levers van muizen verlaagde, als gevolg van oxidatieve stress en waar LPS de hepcidine concentratie sterk deed verhogen. Concluderend kan het gebruik van PCLS nuttig zijn om eiwitten in de urine te identificeren als eiwitten die van de lever afkomstig zijn. Bovendien kan het analyseren van het eiwitprofiel een veelbelovende en niet-invasieve methode zijn voor de identificatie van biomarkers *in vitro* / *ex vivo* (bv in medium van PCLS) of *in vivo* (in urine) en hepcidine zou kunnen dienen als een potentiële nieuwe *in vitro* / *ex vivo* biomarker voor DILI.

Hoofdstuk 4 en **hoofdstuk 5** richten zich op de ontwikkeling van een ex vivo model met het gebruik van PCLS om idiosyncratische DILI in PCLS van muis en mens te detecteren. We ontwikkelden ons model gebaseerd op de inflammatoire stress hypothese, geïntroduceerd door Roth et al., waarbij gebruik gemaakt wordt van gelijktijdige toediening van het geneesmiddel met LPS. We onderzochten de effecten van zowel LPS op de toxiciteit van de geneesmiddelen als de effecten van de geneesmiddelen op de LPS-geïnduceerde ontstekingsreacties. Vijf middelen die reactieve metabolieten produceren en bekend zijn om het feit dat ze idiosyncratische DILI veroorzaken bij mensen werden geselecteerd om getest te worden in ons model. Deze medicijnen waren ketoconazol (KC), clozapine (CZ), diclofenac (DF), troglitazon (TGZ) en carbamazepine (CBZ). Na 24 uur incubatie vertoonden zowel muizen als humane PCLS geïncubeerd met LPS plus KC

of LPS plus CZ, synergistische toxiciteit gebaseerd op ATP niveau, histomorfologie en (alleen in muis) lekkage van lactaat dehydrogenase (LDH). Als controle onderzochten we voriconazol (VC), een geneesmiddel dat dezelfde farmacologische werking heeft als KC, maar dat geen idiosyncratische DILI veroorzaakt in patiënten. Dit middel bleek niet toxisch in muizen PCLS of in ratten in vivo, maar het is bekend dat het een dosisafhankelijke, niet idiosyncratische levertoxiciteit veroorzaakt in mensen die optreedt in ongeveer 51% van de patiënten. Ondanks VC-geïnduceerde toxiciteit, leidde co-incubatie met LPS en VC niet tot synergistische toxiciteit. Ook veroorzaakte incubatie van olanzapine (OZ), het niet-toxische analogon van CZ, tegelijk met LPS ook geen synergistische toxiciteit. Dit verschijnsel van synergistische toxiciteit ging gepaard met een gelijktijdige afname van glutathion (GSH). De andere drie geneesmiddelen die idiosyncratische DILI veroorzaken in mensen, DF, TGZ en CBZ, lieten geen synergistische toxiciteit zien met LPS na 24 uur incubatie in zowel PCLS van muis als mens en geen vermindering van GSH. We hebben ook het effect op de LPS-geïnduceerde cytokineproductie bestudeerd, omdat cytokines een rol zouden kunnen spelen in de mechanismen van idiosyncratische toxiciteit en bovendien wilden we onderzoeken of ze kunnen worden gebruikt als potentiële biomarkers voor idiosyncratische DILI. Geen van de geneesmiddelen ïnduceerde significante veranderingen in de uitscheiding van een van de gemeten cytokines, terwijl LPS de afgifte van de volgende cytokines significant verhoogde: interferon gamma (IFN-γ), interleukine-1beta (IL-1β), interleukine-6 (IL-6), tumor necrose factor (TNF), CC chemokine ligand 3 (CCL3), CC chemokine ligand 5 (CCL5), granulocyt kolonie-stimulerende factor (G-CSF) en granulocyt-macrofaag kolonie-stimulerende factor (GM-CSF). Onze strategie was om naar gemeenschappelijke verschijnselen te zoeken in de LPS geïnduceerde synergistische toxiciteit van KC en CZ. In PCLS van de muis, waren KC en CZ de enige twee middelen die de LPS-geïnduceerde afgifte van G-CSF en GM-CSF verhoogden. Maar dit was niet het geval in humane PCLS waar TNF de enige cytokine was die verder verhoogd werd door KC en CZ en niet door de andere geneesmiddelen na 24 uur incubatie. Onze bevinding over TNF in humane PCLS kwam overeen met die van anderen die meldden dat TNF een cruciale rol speelt in inflammatoire stress-gerelateerde idiosyncratische DILI mechanismen van aflatoxine B1, trovafloxacine, en ranitidine in ratten en muizen *in vivo*. Deze gegevens suggereren dat zowel TNF als GSH gebruikt kunnen worden als voorspellende biomarkers van inflammatoire stressgerelateerde idiosyncratische DILI in humane PCLS en mogelijk ook in andere *in vitro* / *ex vivo* systemen. Bovendien kan GSH ook als translationele biomarker dienen in muizen *in vitro, ex vivo* en misschien zelfs *in vivo*. Ondanks vergelijkbare bevindingen in het synergistische toxiciteit fenomeen gerelateerd aan LPS + KC en LPS + CZ in zowel muizen als humane PCLS, waren er belangrijke verschillen tussen muis en

mens in hoe deze geneesmiddelen de LPS-geïnduceerde afgifte van cytokines beïnvloeden. Dit suggereert dat de mechanismen van idiosyncratische levertoxiciteit die betrokken zijn bij de muis en mogelijk verschillend zijn van die van de mens.

Het was redelijk onverwacht om alleen synergistische toxiciteit te vinden in 2 van de 5 idiosyncratische geneesmiddelen, omdat ze allemaal bekend zijn als veroorzakers van IDILI in de mens. Vandaar dat we in **hoofdstuk 4** onderzocht hebben of er een correlatie bestaat tussen de incubatietijd en de observatie van synergistische toxiciteit in PCLS van muizen. We vonden dat twee van de idiosyncratische DILI-gerelateerde medicijnen die geen synergistische toxiciteit met LPS veroorzaakten na 24 uur incubatie, namelijk DF en TGZ, wel synergistische toxiciteit vertoonden (gebaseerd op ATP) met LPS na 48 uur incubatie van muizen PCLS. Maar zelfs na 48 uur verhoogde LPS nog steeds niet de toxiciteit van CBZ in muizen PCLS. Toekomstig onderzoek zal erop gericht zijn om deze synergistische toxiciteit na 48 uur verder te karakteriseren.

In **hoofdstuk 5** hebben we de termen 'responders' (synergistische toxiciteit waargenomen in een individueel weefselmonster) en 'niet-responders' (geen synergistische toxiciteit waargenomen in een individueel weefselmonster) geïntroduceerd. Hoewel de totale effecten van LPS + KC en LPS + CZ significant waren voor de gehele groep van geteste humane levers, was er een aanzienlijke variatie in de effecten van de interactie van LPS + medicijn, wat er op duidt dat sommige humane levers niet reageerden waar andere levers wel sterk reageerden met synergistische toxiciteit. Zo werd synergistische toxiciteit waargenomen bij 7 van de 10 humane levers (70% responders) behandeld met LPS + KC en in 9 van de 14 humane levers (64% responders) behandeld met LPS + CZ. Anderzijds werd slechts 1 van de 8 muizen als een niet-responder beschouwd. Interessant is dat we vonden dat de relatieve hoeveelheden van zowel het glutathionconjugaat (CZ-GSH) en cysteïneconjugaat (CZ-Cys) van CZ significant lager waren onder de responders in hPCLS geïncubeerd met LPS in vergelijking met die zonder LPS. Onder de niet-responders werden er geen verschillen in de hoeveelheid van deze metabolieten waargenomen in de aan- of afwezigheid van LPS. De lagere hoeveelheden van CZ-SG en CZ-Cys in de responder groep na co-incubatie met LPS en CZ kan worden toegeschreven aan het lagere GSH niveau van deze groep PCLS, wat zou kunnen resulteren in een grotere hoeveelheid reactieve metabolieten van CZ wat weer leidt tot hogere toxiciteit die veroorzaakt zou kunnen worden door de covalente binding van deze reactieve metabolieten met eiwitten.

Samenvattend, hebben wij een *ex vivo* model ontwikkeld met gebruik van ratten, muizen en humane PCLS dat in staat is de intrinsieke (APAP geïnduceerde) DILI en idiosyncratische DILI gerelateerd aan de inflammatoire stress hypothese te detecteren. Dit *ex vivo* PCLS model wordt als een meer efficiënt model beschouwd in vergelijking met

in vivo modellen. Tegelijkertijd lijkt het ook een beter representatief model dan andere *in vitro* modellen, omdat in PCLS alle celtypes van de lever in hun natuurlijke omgeving aanwezig zijn, wat belangrijk is vanwege de mogelijkheid om de effecten van cytokines uitscheidingen de metabolieten geproduceerd door de PCLS te identificeren. Bovendien is de mogelijkheid om interindividuele verschillen waar te nemen met PCLS belangrijk om gevoelige individuen te identificeren. Een ander belangrijk voordeel is dat PCLS een robuust translationeel model kan zijn met de mogelijkheid om leverweefsel van dieren en mensen te gebruiken en hun bevindingen te vergelijken. Onze studies hebben niet alleen significante verschillen tussen ratten, muizen en humane PCLS in hun gevoeligheid voor de toxiciteit van geneesmiddelen aangetoond, maar ook prominent soortverschillen gedetecteerd in cytokine afgifte na LPS stimulatie en LPS + geneesmiddel behandeling. We identificeerden hepcidine als translationele biomarker voor intrinsieke DILI in PCLS en hepcidine kan ook verder worden onderzocht als *in vivo* DILI biomarker bij de mens. GSH kan een andere potentiële translationele biomarker zijn dat gemeten kan worden in *in vitro* en *ex vivo* systemen bij gelijktijdige blootstelling aan LPS en geneesmiddel en ook in dieren *in vivo*. Bovendien kan TNF verder worden onderzocht als een *in vitro* / *ex vivo* biomarker voor inflammatoire stress-gerelateerde idiosyncratische DILI in mensen maar niet in muizen. De soortverschillen in toxiciteit, metabolisme, en de effecten op LPS-geïnduceerde cytokine afgifte onderstrepen het belang van experimenten met humaan weefsel of humane cellen in aanvulling op dierexperimenten om de mechanismen van intrinsieke en idiosyncratische DILI in de mens te ontrafelen. Bovendien draagt het gebruik van PCLS bij tot de vermindering van het aantal dieren dat gebruikt wordt in onderzoek, omdat uit een lever van een individueel dier vele PCLS kunnen worden gemaakt (~ 40 uit een muizenlever en ~ 200 uit een rattenlever) die gebruikt kunnen worden om meerdere behandelcondities te testen. Evenzo zal het gebruik van humane PCLS ook kunnen bijdragen aan de vermindering en uiteindelijke vervanging van dierexperimenten in de toxicologie.

Concluderend kan het PCLS systeem een robuust en veelbelovend *ex vivo* translationeel screenings instrument zijn, dat kan worden gebruikt in preklinische experimenten om humaan-specifieke intrinsieke en idiosyncratische DILI in een vroeg stadium van de geneesmiddelontwikkeling te identificeren en karakteriseren.

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For all other PhDs in the group (Na Guan, Suresh Vatakuti, Ming Li, Adriana Mattos, Marlies Schippers, Carian Boorsma, Christina Draijer, Karin de Ruiter, Amirah Adlia). Good luck also for finishing your PhDs. Na, good luck for your defense. Suresh and Ming, good

luck on supervising your first student. Adriana and Marlies, hope that you will submit your thesis very soon. For Carian, the tallest person in our group (note: 'person' not only 'female'), thanks for joining the pub quizzes sometimes. For Marlies, Carian, Christina, and Karin, thanks so much for organizing the group borrel. It's always so fun. Amirah, the new girl, good luck!

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Mackenzie

P.S. For all the people reading this sentence, from now on it's Dr. Hadi to you :-p